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14. ABSTRACT

We show that MCF-10A cells overexpressing ErbB2 upregulate integrin α5 via the MAPK pathway in 3D acini and found elevated integrin α5 levels associated with ErbB2 status in human breast cancer. Moreover, integrin α5 is required for ErbB2-mediated anoikis resistance and for optimal ErbB2 signaling to the Mek-Erk-Bim axis as depletion of integrin α5 reverses anoikis resistance and Bim inhibition. Integrin α5 is required for full activation of ErbB2 tyrosine phosphorylation on Tyr-877 and ErbB2 phosphorylation is associated with increased activity of c-Src in the absence of cell adhesion. We show that blocking elevated c-Src activity during cell detachment reverses ErbB2-mediated survival and Bim repression. Integrin α5 serves as a key mediator of c-Src and ErbB2-survival signaling under states of low adhesion necessary to block the pro-anoikis mediator Bim and suggest this pathway represents a potential novel therapeutic target in ErbB2-positive tumors to reverse anoikis resistance.

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Introduction

Recent evidence suggests that integrin receptors are required for oncogenic phenotypes in vivo (Guo et al., 2006; White et al., 2004). Thus, it is not clear whether growth factor receptors expressed at high levels still require integrins in the absence of adhesion for proper survival signaling and anoikis resistance. We have previously shown that cells overexpressing the receptor tyrosine kinase, ErbB2, can induce anoikis resistance in mammary epithelial cells (Reginato et al., 2003). In addition, activation of ErbB2 in 3D acini leads to increase in proliferation and luminal filling (Muthuswamy et al., 2001). ErbB2 inhibition of luminal apoptosis during morphogenesis is associated with increased Erk activation and decreased Bim expression (Reginato et al., 2005). It remains unclear whether epithelial cells overexpressing ErbB2 still require integrins for anoikis resistance. Our current work examines the involvement of the integrin α5 subunit in ErbB2 mediated oncogenesis.

Body

We first compared MCF10A control cells (pBabe) to MCF10A cells overexpressing ErbB2 (NeuN) to determine whether there was differential expression of integrin subunits. We found that integrin α5 was specifically upregulated in ErbB2-overexpressing cells both at the mRNA and protein levels, whereas levels of integrins α2, α3, α6, β1 and β4 remained unchanged. We also determined that protein levels of integrin α5 were elevated in NeuN cells as compared to Babe cells during 3D morphogenesis, and are further elevated in detached cells versus attached cells. To determine whether integrin α5 expression correlates with ErbB2 positive breast cancer tissue, we performed immunohistochemical staining on ErbB2 positive breast cancer tissue using breast cancer tissue arrays (94 cases total). We found very little staining of integrin α5 in normal breast epithelial cells, benign breast tissue as well as ErbB2-negative malignant tissue. We found 68% of ErbB2 positive cancer contained elevated integrin α5 staining. A two-tailed Pearson's correlation showed that there is a significant and positive association between integrin α5 and ErbB2 expression. These results indicate that ErbB2 upregulates integrin α5 RNA and protein in vitro and increased integrin α5 expression is associated with ErbB2 positive breast cancers.

We next examined whether integrin α5 had a functional role during ErbB2-mediated oncogenesis. We found that reducing integrin α5 levels by RNAi induces luminal apoptosis in ErbB2-overexpressing 3D acinar structures, whereas treatment with control siRNA targeting luciferase did not induce apoptosis of luminal cells. Similarly, treatment with RNAi targeting integrin α5 sensitized ErbB2 cells to anoikis as well as decreased their ability to form colonies in soft agar, thus indicating that reducing integrin α5 levels leads to a decrease in the oncogenic potential of ErbB2. Additionally, we looked at signaling from the ErbB2 receptor in suspension cultures and found that when integrin α5 levels are reduced via RNAi, ErbB2 signaling through the MAP kinase pathway is inhibited. Specifically, levels of phosphorylated Erk are decreased, whereas Bim expression and Caspase-3 cleavage are increased, thus indicating an increase in anoikis. More interestingly, we observed a decrease in total phosphorylation of the ErbB2 receptor itself, and a particular decrease at Tyrosine877, previously shown to be regulated by c-Src (Xu et al., 2007). Further studies revealed that decreasing integrin α5 levels resulted in a decrease in c-Src phosphorylation at Tyrosine416 in both control cells as well as ErbB2 cells attached to tissue culture plates or cultured in suspension. We also determined that both chemical inhibition as well as treatment with shRNA targeting c-Src resulted in a decrease in the oncogenic potential of ErbB2, similar to the phenotype observed when we targeted integrin α5 with RNAi. Our findings therefore show that integrin α5 is required for maintaining elevated c-Src activity contributing to ErbB2 phosphorylation and downstream Mek/Erk-mediated inhibition of Bim during loss of adhesion. Thus, the integrin α5 and c-Src regulation of ErbB2-Erk axis may be a vital cascade for developing novel strategies for cancer therapy.

Key Research Accomplishments

- Established that integrin α5 is upregulated in ErbB2 positive breast cancers

- Demonstrated that ErbB2 requires integrin α5 for its inhibition of anoikis resistance as well as luminal apoptosis in 3D acinar structures
- Demonstrated that integrin α5 is required for proper ErbB2 receptor activation and downstream signaling, ErbB2-mediated inhibition of the proapoptotic molecule, Bim, as well as ErbB2-mediated anchorage independent growth
- Identified the involvement of c-Src in integrin α5 regulation of ErbB2 function in suspension and 3D morphogenesis of MCF-10A cells overexpressing ErbB2

Reportable Outcomes

Manuscript titled "ErbB2 requires integrin α5 for anoikis resistance via c-Src regulation of receptor activity in human mammary epithelial cells" originally submitted to Journal of Cell Science on February 23, 2009 and resubmitted to Journal of Cell Science on August 18, 2009.

Conclusion

Overexpression of growth factor receptors, which is found in many epithelial carcinomas, is thought to uncouple and bypass the requirement for integrin signaling in the absence of adhesion. Our results here reveal that growth factor receptors can regulate specific integrin subunits to maintain their own activation and thus remain highly dependent on integrin function under conditions of no or reduced matrix adhesion. The integrin α5/c-Src survival pathway examined in these studies may be specific for RTKs since we also found that reducing integrin α5 had no effect on anchorage independent growth of Ras^{G12V} overexpressing cells. Therefore, elevating integrin α5 under low adhesion may be a general mechanism of survival from anoikis that is co-opted by RTKs in cancer cells to maintain their own activity, promote cell survival and block luminal clearing in cases where tissue architecture and cell-matrix interactions are compromised possibly in early stages of cancer. Other recent studies showed that maintenance of c-Src activation in suspended prostate cancer cells required production of reactive oxygen species (ROS) (Giannoni et al., 2009). It is not clear whether ROS plays a role in c-Src regulation during anoikis in MCF-10A cells. However, some of our further studies will involve determining whether integrin α5 plays a role in ROS regulation of c-Src during cell detachment.

References

1. **Giannoni, E., Fiaschi, T., Ramponi, G. and Chiarugi, P.** (2009). Redox regulation of anoikis resistance of metastatic prostate cancer cells: key role for Src and EGFR-mediated pro-survival signals. *Oncogene* **28**, 2074-86.
2. **Guo, W., Pylayeva, Y., Pepe, A., Yoshioka, T., Muller, W. J., Inghirami, G. and Giancotti, F. G.** (2006). Beta 4 integrin amplifies ErbB2 signaling to promote mammary tumorigenesis. *Cell* **126**, 489-502.
3. **Muthuswamy, S. K., Li, D., Lelievre, S., Bissell, M. J. and Brugge, J. S.** (2001). ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nat Cell Biol* **3**, 785-92.
4. **Reginato, M. J., Mills, K. R., Paulus, J. K., Lynch, D. K., Sgroi, D. C., Debnath, J., Muthuswamy, S. K. and Brugge, J. S.** (2003). Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. *Nat Cell Biol* **5**, 733-40.
5. **Reginato, M. J. and Muthuswamy, S. K.** (2006). Illuminating the center: mechanisms regulating lumen formation and maintenance in mammary morphogenesis. *J Mammary Gland Biol Neoplasia* **11**, 205-11.
6. **White, D. E., Kurpios, N. A., Zuo, D., Hassell, J. A., Blaess, S., Mueller, U. and Muller, W. J.** (2004). Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. *Cancer Cell* **6**, 159-70.
7. **Xu, W., Yuan, X., Beebe, K., Xiang, Z. and Neckers, L.** (2007). Loss of Hsp90 association up-regulates Src-dependent ErbB2 activity. *Mol Cell Biol* **27**, 220-8.

ErbB2 requires integrin α5 for anoikis resistance via c-Src regulation of receptor activity in human mammary epithelial cells

Short Title: ErbB2 requires Integrin α5 for survival

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Summary

ErbB2, a receptor tyrosine kinase highly expressed in many epithelial tumors, is known to inhibit apoptotic signals. Overexpression of ErbB2 causes anoikis resistance that contributes to luminal filling in three-dimensional (3D) mammary epithelial acinar structures in vitro. Given that integrin and growth factor receptors are highly interdependent for proper function, we examined the role, if any, of integrin subunits in ErbB2-mediated survival signaling. Here, we show that MCF-10A cells overexpressing ErbB2 upregulates integrin α5 via the MAP-kinase pathway in 3D acini and found elevated integrin α5 levels associated with ErbB2 status in human breast cancer. Moreover, integrin α5 is required for ErbB2-mediated anoikis resistance and for optimal ErbB2 signaling to the Mek-Erk-Bim axis as depletion of integrin α5 reverses anoikis resistance and Bim inhibition. Integrin α5 is required for full activation of ErbB2 tyrosine phosphorylation activity on Tyr-877 and ErbB2 phosphorylation is associated with increased activity of c-Src in the absence of cell adhesion. Indeed, we show that blocking elevated c-Src activity during cell detachment reverses ErbB2-mediated survival and Bim repression. Thus, integrin α5 serves as a key mediator of c-Src and ErbB2-survival signaling under states of low adhesion necessary to block the pro-anoikis mediator Bim and suggest this pathway represents a potential novel therapeutic target in ErbB2-positive tumors to reverse anoikis resistance.

Introduction

Cell to extracellular matrix (ECM) adhesion plays a central role for the structural organization of tissues of multicellular organisms. Adhesion mediated regulation of cell survival contributes to maintenance of tissue homeostasis by allowing cells to remain in their proper tissue environment. Absence of survival signals from ECM can trigger apoptosis in a process referred to as anoikis (Frisch and Screaton, 2001). Tumor cells become anchorage-independent for growth and survival and thus most epithelial-derived cancer cells display significant resistance to anoikis (Schwartz, 1997). This characteristic has important implications for metastasis as cells that survive after detachment from their primary site may travel through circulatory systems to secondary sites (Simpson et al., 2008). Studies using three dimensional (3D) organotypic culture systems, which have been increasingly used to understand pathways regulating glandular morphogenesis and epithelial cancer development (Debnath and Brugge, 2005), have implicated anoikis resistance as an early event in cancer progression. Many types of early breast cancer lesions such as ductal carcinoma in situ are characterized by filling of the luminal space (Harris, 2004). Mechanisms underlying lumen formation have been examined using MCF-10A cells, an immortalized human mammary epithelial cell line. These cells when cultured in reconstituted basement membrane, undergo a series of morphogenic changes resulting in formation of acinus-like structures containing a single layer of polarized cells surrounding a hollow lumen (Muthuswamy et al., 2001). Clearance of centrally located cells in this model involves apoptosis, because these cells within acinar structures are not in direct contact with matrix suggesting that anoikis may be involved (Debnath et al., 2002). Indeed, we have shown that cell detachment at the single cell level and apoptosis during lumen formation in 3D morphogenesis both require induction of the Bcl-2-family proapoptotic BH3-only protein Bim; and

oncogenes that induce anoikis resistance and are capable of blocking or delaying lumen formation in 3D culture block Bim induction (Reginato et al., 2003); (Reginato et al., 2005); (Reginato and Muthuswamy, 2006). Recent studies have found that Bim is also required in the mouse mammary gland for apoptosis during lumen formation (Mailleux et al., 2007) correlating with in vitro 3D acinar morphogenesis culture system.

Inhibition of Bim expression in MCF-10A cells is coordinated by both integrin and EGF receptor regulation of Erk signaling that is dependent on cell adhesion. Loss of integrin receptor adhesion leads to downregulation of EGF receptor expression and signaling to the Mek/Erk pathway leading to induction of Bim (Reginato et al., 2003). Cells overexpressing EGF receptor are able to escape regulation from loss of integrin engagement, additionally they allow cell survival from anoikis and inhibit Bim expression; suggesting that elevated levels of growth factor receptor may be able to uncouple from integrin regulation. However, recent evidence suggests that integrin receptors are required for oncogenic phenotypes in vivo (Guo et al., 2006; White et al., 2004). Thus, it is not clear whether growth factor receptors expressed at high levels still require integrins in the absence of adhesion for proper survival signaling and anoikis resistance.

ErbB2 (HER2/Neu) is a receptor tyrosine kinase (RTK) that is overexpressed in 20% to 30% of invasive breast tumors and up to 85% of comedo type ductal carcinoma in situ (DCIS), a premalignant stage of breast cancer (Hynes and Stern, 1994; Lohrisch and Piccart, 2001). A number of ErbB2 inhibitors, although initially effective in breast cancer patients, lose activity during prolonged treatment (Nahta et al., 2006) indicating the need for identification of additional therapeutic targets. We have previously shown that cells overexpressing ErbB2 can induce anoikis resistance in mammary epithelial cells (Reginato et al., 2003). In addition, activation of ErbB2 in 3D acini leads to increase in proliferation and luminal filling (Muthuswamy et al., 2001). ErbB2 inhibition of luminal apoptosis during morphogenesis is associated with

increased Erk activation and decreased Bim expression (Reginato et al., 2005). It remains unclear whether epithelial cells overexpressing ErbB2 still require integrins for anoikis resistance. Recently, it was shown that mice expressing a dominant negative form of integrin β4 could inhibit ErbB2-mediated mammary oncogenesis suggesting that cancer cells overexpressing RTKs are dependent on integrin beta subunits for their growth and survival (Guo et al., 2006). However, the requirement of specific integrin subunits for ErbB2-mediated anoikis resistance has not been addressed.

Here we show that ErbB2 expressing breast cancers positively correlate with increased integrin α5 levels and ErbB2 requires integrin α5 for anoikis resistance. Integrin α5 is highly upregulated in ErbB2-overexpressing cells via regulation of the Mek/Erk kinase pathway. Additionally, integrin α5 is required for ErbB2-mediated anoikis resistance both in 3D acini and soft agar colony formation. Reducing expression of integrin α5 in ErbB2-overexpressing cells leads to loss of c-Src hyperactivity in the absence of adhesion. Furthermore, this reduced expression was found to decrease ErbB2 tyrosine phosphorylation on Y877 and decreased signaling to the Mek/Erk pathway allowing reversal of Bim inhibition. Elevation of integrin α5 in epithelial cells with low adhesion helps activate and couple c-Src activity to RTKs such as ErbB2 and allow survival from anoikis. Thus, a signaling loop involving integrin α5, c-Src, RTK, and Erk is necessary for inhibition of Bim expression and anoikis resistance; which suggests this loop may represent a potential novel therapeutic target in ErbB2-positive tumors to reverse anoikis resistance. Moreover, our data indicates that tyrosine kinase receptors still require integrins for proper survival signaling even in conditions of suboptimal matrix interaction.

Results

Integrin α5 expression is upregulated by ErbB2 expressing cells and elevated in ErbB2 positive breast cancers.

To determine whether ErbB2 regulates specific integrin subunits in human mammary epithelial cells we examined RNA expression of a number of integrin subunits in MCF-10A cells stably overexpressing wildtype ErbB2 (pBabe-NeuN) compared to vector alone (pBabe). By using an RNase protection assay (RPA) integrin probe set, we found a number of integrin subunits which are constitutively expressed in normal MCF-10A cells and cells overexpressing ErbB2. MCF-10A cells overexpressing ErbB2 did not significantly alter RNA expression of most integrin subunits compared to control MCF-10A cells. In contrast, we found a significant and specific upregulation of the integrin α5 subunit in cells overexpressing ErbB2 (data not shown). We validated RPA results using quantitative RT-PCR and found that integrin α5 mRNA was elevated three-fold in ErbB2 overexpressing cells compared to parental MCF-10A cells (Fig 1A). We did not detect changes in other integrin alpha subunit mRNA expression including α2 and α6 by QRT-PCR. In addition, we also examined expression of these integrin subunits in MCF-10A cells overexpressing constitutively active ErbB2 (NeuT) and found an eight-fold elevation of integrin α5 mRNA but no change in α2 or α6 levels compared to control MCF-10A cells. Consistent with RNA expression, integrin α5 protein levels were increased two-fold in ErbB2 (NeuN or NeuT) overexpressing cells compared to control cells (Fig. 1B) and analysis by flow cytometry showed a two to three-fold increased membrane localization of integrin α5 in ErbB2 overexpressing cells (Supplementary Fig. 1). We found no increase in protein expression of integrin α3, integrin β4, or integrin β1 in ErbB2 overexpressing cells compared to control cells (Fig. 1B) and also no change in surface expression of integrin α6 or integrin β1 (data not shown).

To determine whether ErbB2 overexpression in MCF-10A cells undergoing 3D morphogenesis also increased expression of integrin α5, we analyzed levels of several integrin subunits during morphogenesis. Cells stably overexpressing wildtype ErbB2 (NeuN) had three to four-fold higher levels of integrin α5 at day 6 and day 8 when compared to control cells (Fig. 1C). Expression of other integrin subunits was not

increased including integrin α3 or integrin β1 during morphogenesis. We have previously shown that normal cells undergoing morphogenesis in 3D culture induces Bim expression around day 8, and that Bim is required for clearing of the central lumen (Reginato et al., 2005). Bim inhibition is associated with luminal filling and deceased apoptosis in cells overexpressing oncogenes, including ErbB2, during morphogenesis (Reginato et al., 2005) (Fig. 1C). We also examined localization of integrin α5 in normal and ErbB2-expressing acini structures by immunostaining. As previously shown (Muthuswamy et al., 2001), integrin α6 is localized primarily at the basal surface with weak staining at lateral surface of normal MCF-10A acini (Fig. 1D). However, unlike integrin α6, integrin α5 is localized predominantly on lateral surface of normal acini (Fig. 1D, arrowed). In ErbB2 expressing acini, organization and polarity of acini structures are disrupted as previously described (Muthuswamy et al., 2001) (Aranda et al., 2006) and integrin α5 localization is no longer predominantly at lateral surface.

To determine whether integrin α5 expression correlates with ErbB2 positive breast cancer tissue, we performed immunohistochemical staining on ErbB2/HER2 positive breast cancer tissue using breast cancer tissue arrays. We found very little staining of integrin α5 in normal breast epithelial cells, benign breast tissue as well as ErbB2-negative malignant tissue (Fig. 2A). However, we found 68% of ErbB2 positive cancer contained elevated integrin α5 staining (Figure 2A, Supplementary Table I). Statistical analysis showed that there is a significant and positive association between integrin α5 and ErbB2/HER2 expression (Pearson's correlation= 0.219, two-tailed, $p<0.05$) (Fig. 2B). These results indicate that ErbB2 upregulates integrin α5 RNA and protein in vitro and increased integrin α5 expression is associated with ErbB2 positive breast cancers.

Integrin α5 is required for ErbB2-mediated inhibition of luminal apoptosis and anoikis resistance.

Upregulation of integrin α5 by ErbB2 suggests that integrin α5 may contribute to ErbB2-oncogenic phenotypes including anchorage-independence. We have previously shown a critical interdependence between integrin and growth factor receptors in regulation of normal epithelial cell survival during detachment from matrix signals (Reginato et al., 2003). Thus, we hypothesized that oncogenes, such as ErbB2, that inhibit anoikis may co-opt specific ECM-related molecules that contributes to survival in absence of adhesion. To determine whether integrin α5 is required for ErbB2-anoikis resistance, we used RNAi to reduce expression of integrin α5. ErbB2 cells transfected with two separate small interfering (siRNA) oligonucleotides homologous to integrin α5 sequence, but not control siRNA, showed significantly reduced expression of integrin α5 at day 0 of morphogenesis assay (Fig. 3A). Expression of other integrin subunits such as integrin α3 or integrin β1 was not affected by the integrin α5 siRNA transfection (data not shown). Luminal apoptosis was measured by determining the percent of acini with centrally localized cleaved caspase-3 staining. Reducing integrin α5 expression significantly increased luminal apoptosis in ErbB2 expressing acini as measured by percentage of caspase-3 positive acini (Fig. 3B) compared to cells transfected with control siRNA. At day 10 and 12 of morphogenesis, acini derived from cells transfected with integrin α5 siRNA oligos contained two or three-fold higher centrally localized, activated caspase-3-positive staining compared to acini transfected with control siRNA (Fig. 3C). In addition to control siRNA, we also found no effect of reducing expression of integrin α3 on luminal apoptosis (data not shown). Thus, integrin α5 expression is specifically required for ErbB2-mediated inhibition of luminal apoptosis.

Since luminal apoptosis during morphogenesis is associated with anoikis, we sought to determine the role of integrin α5 in ErbB2-mediated anoikis resistance. Following 48 hours of cell detachment, anoikis sensitivity was increased two-fold in ErbB2-overexprressing cells transfected with integrin α5 siRNA oligonucleotides compared to cells transfected with control oligos (Fig. 4A). Consistent with this data, increased apoptosis in ErbB2 cells targeted with integrin α5 RNAi was associated with increased levels of

cleaved caspase-3 (Fig. 4B). We have previously shown that ErbB2 blocks anoikis in part by inhibiting expression of the proapoptotic protein Bim during cell detachment (Reginato et al., 2003). Accordingly, we examined Bim expression in cells targeted with integrin α5 siRNA. ErbB2 overexpressing cells targeted with integrin α5 siRNA, but not control siRNA (Fig. 4B) or integrin α3 siRNA (data not shown), caused a significant increase of Bim expression in cells in suspension. Although targeting integrin α5 led to significant increase in luminal apoptosis in 3D and increased anoikis sensitivity in suspended cells, we did not detect any evidence of apoptosis in ErbB2 cells attached in 2D culture (Fig. 4A) or in outer cells in direct contact with ECM in 3D culture (Fig. 3B). Decreasing integrin α5 levels did not alter sensitivity of ErbB2-overexpressing cells to other apoptotic stimuli including U.V. or hydrogen peroxide treatment (data not shown) in 2D; suggesting that the relationship between ErbB2 and integrin α5 and apoptosis is specific to cells under low adhesion. Moreover, reducing integrin α5 in ErbB2 overexpressing cells did not alter overall cell growth in 3D conditions (Supplementary Fig. S2A). or in 2D attached cells (Supplementary Fig. S2B). Although reducing integrin α5 levels increased luminal apoptosis in ErbB2 overexpressing acini, we found that it only partially led to hollowing the lumen of these acini (data not shown). Since targeting intgerin α5 had minimal effects on growth in 3D of ErbB2 cells its possible these acini remain partially filled, in spite of increasing apoptosis, as they remain highly proliferative.

We also examined the role of integrin α5 in parental MCF-10A cells. We found that cell detachment of MCF-10A cells also resulted in elevation of integrin α5 protein levels (Supplementary Fig. S3A) and reducing integrin α5 levels with RNAi sensitized cells to anoikis, increased levels of Bim and cleaved caspase-3 when placed in suspension compared to control RNAi cells (Supplementary Fig. S3) suggesting integrin α5 may play a general role in epithelial anoikis sensitivity. As in ErbB2-overexpressing cells, reducing intgerin α5 levels in parental MCF-10A cells did not alter growth or organization in 3D culture (Supplementary Fig. S3D). Thus, integrin α5 is critical for anoikis sensitivity of both normal MCF-10A

cells and those overexpressing ErbB2 and is required for ErbB2-mediated inhibition of the proapoptotic protein Bim in the absence of matrix attachment but does not appear to affect cell growth.

Anoikis resistance is critical for anchorage independence at the single cell level. We thus also examined the effect of reducing integrin α5 expression on ErbB2-mediated anchorage independent colony formation. For these experiments we used cells overexpressing the active form of ErbB2 (NeuT) as they form robust colonies in soft agar. We found that NeuT overexpressing cells targeted with integrin α5, but not control siRNA, formed fewer colonies in soft agar by greater then two-fold (Fig. 4C). Targeting integrin α3 had no effect on colony formation of NeuT cells in soft agar (data not shown). Thus, integrin α5 is specifically required for ErbB2-mediated anchorage independence. To verify specificity of RNAi oligos, ErbB2 overexpressing cells were infected with shRNA against integrin α5 and, when compared to scrambled shRNA, was also able to reverse anoikis resistance, Bim inhibition, and colony formation in soft agar (Supplementary Fig. S4 and S5). This effect of integrin α5 on ErbB2 oncogenesis seems to be specific, as reducing integrin α5 levels in MCF-10A cells overexpressing H-Ras^{G12V} cells had little effect on anchorage-independent growth (data not shown). Thus, integrin α5 is required for ErbB2-mediated anoikis resistance in 3D acini and during anchorage independence. Additionally, integrin α5 is required for ErbB2-mediated inhibition of Bim expression.

Role of integrin α5 in ErbB2-mediated signaling.

Oncogenes such as ErbB2 maintain active signaling pathways in the absence of matrix attachment, which can regulate cell survival pathways. Indeed, we have shown that oncogene overexpression is able to maintain the Erk pathway active during cell detachment allowing inhibition of both Bim expression and anoikis (Reginato et al., 2003). Thus, we examined the role of integrin α5 in regulating ErbB2-mediated signaling pathways. Decreased expression of integrin α5 had minor effect on signaling in attached ErbB2

cells (Fig. 5A). However, decreasing integrin α5 expression in ErbB2 expressing cells decreased Mek and Erk activity when cells were placed in suspension (Fig. 5A) in a manner consistent with loss of Bim suppression. To determine the source of altered signaling we examined whether integrin α5 regulated the activation of ErbB2 phosphorylation. Decreasing integrin α5 levels had little effect on ErbB2 phosphorylation in attached cells (Figure 5B). Remarkably, in suspended cells we find that decreasing integrin α5 levels reduces total phosphotyrosine levels of ErbB2 as well a phosphorylation of Tyr-877 and Tyr-1248 without altering total levels of ErbB2 protein (Fig. 5B). Recent studies have shown that c-Src is a key regulator of ErbB2 tyrosine phosphorylation (Ishizawar et al., 2007) specifically at Tyr-877 (Xu et al., 2007) and that c-Src activity is required for ErbB2-mediated soft agar colony formation. We therefore sought to examine changes in c-Src activity in integrin α5 depleted cells. Cells transfected with siRNA against control oligos had low level of c-Src activity in attached cells. However, in cells placed in suspension, c-Src activity increased dramatically. Conversely, cells transfected with two different siRNA oligos against α5 had slightly reduced levels of c-Src activity in attached cells and blocked induction of c-Src activity in cells placed in suspension (Fig. 5A). We did not detect changes in other integrin-mediated pathways such as FAK (data not shown) or Akt in suspended ErbB2 overexpressing cells. ErbB2 overexpressing cells that were infected with integrin α5 shRNA also caused inhibition of total ErbB2 tyrosine activity and reduced c-Src phosphorylation when compared to control shRNA infected MCF-10A cells (Supplementary Fig. S4A). As in MCF-10A cells overexpressing ErbB2, normal MCF-10A cells also elevate c-Src activity when placed in suspension (Supplementary Fig. S3A) and reducing integrin α5 levels blocked activation of Src in the absence of adhesion (Supplementary Fig. S3B). Therefore, we conclude that integrin α5 expression is required for maintaining full ErbB2-tyrosine phosphorylation and ErbB2-mediated Mek/Erk activity during cell detachment, and is also required for general elevated c-Src activity in suspension.

Role of c-Src in ErbB2-mediated anoikis resistance.

We hypothesized that expression of integrin α5 contributes to activation and/or maintenance of active c-Src during cell detachment that could be required for optimal ErbB2 phosphorylation, leading to downstream signaling that allows anoikis resistance through inhibition of Bim. To test this, we examined the role of c-Src in ErbB2-mediated anoikis resistance. Treatment of ErbB2 cells in suspension with c-Src-family kinase inhibitor PP1 or PP2 reversed anoikis resistance by ErbB2 (Supplementary Fig. S6A). Moreover, treatment of ErbB2 cells during anoikis with PP1 or PP2 showed decrease of ErbB2 Tyr-877 phosphorylation, increased levels of Bim expression by six-fold, and elevated cleaved caspase-3 (Supplementary Fig. S6B). Treatment of attached ErbB2 overexpressing cells with PP1 or PP2 had no effect on apoptosis or Bim expression (data not shown). We also tested the effect of c-Src inhibitors on ErbB2-overexpressing 3D acini. Starting at day 8, acini were treated for 48 hrs with c-Src inhibitor PP2, and analyzed for cleaved caspase-3 positivity at day 10 and day 12. We observed an increase in cleaved caspase-3 staining within centers of acini treated with PP2 (Fig. 6A). When we ascertained the percentage of cleaved caspase-3 positive acini in both control and PP2-treated cultures, we found at least a two-fold increase in luminal cell death in PP2-treated cells vs. control (Fig. 6B). As was the case in cells targeted with integrin α5 RNAi, the cells undergoing apoptosis due to treatment with c-Src inhibitor were predominantly in the center of each acini (Fig. 6A). We also sought to determine if reversal of ErbB2-mediated protection from luminal apoptosis in 3D acini by c-Src inhibitor PP2 correlated with Src activity, ErbB2 Tyr-877 phosphorylation, and Bim expression. Indeed, 3D acini treated with PP2 was associated with decreased phosphorylation of c-Src on Tyr-416, ErbB2 on Tyr-877 and increased levels of Bim expression (Fig. 6C). To ensure specificity of c-Src inhibitors, we targeted c-Src with RNAi and found that reducing expression of c-Src also blocked ErbB2 activation, reversed Bim inhibition in suspended cells (Figure 5D) and increased sensitivity to

anoikis (Figure 6E) similar to effects of c-Src inhibitors. Thus, decreasing c-Src activity in ErbB2-overexpressing cells leads to decrease in ErbB2 activation and reversal of anoikis resistance via upregulation of Bim expression, and negatively affects ErbB2-mediated survival signaling in a manner similar to reduction of integrin α5 expression.

ErbB2 regulation of integrin α5 via Mek/Erk pathway. To characterize the ErbB2-mediated regulation of integrin α5 expression, we examined the effects of inhibiting several kinases downstream of ErbB2 on integrin α5 expression in 3D culture. Incubation of ErbB2-expressing acini for 48 hrs with a MEK inhibitor (UO126) blocked integrin α5 expression (Fig. 7A) in 3D culture suggesting that signaling downstream of Mek regulates integrin α5 expression. Treatment of acini with PI(3)K inhibitor (LY294002) had no effect on integrin α5 levels (Fig. 7A). To determine whether activation of the Erk pathway is sufficient to upregulate integrin α5 in 3D acini, stable pools of MCF-10A cells expressing a constitutively activate variant of Mek (MEK2-DD) were generated and cultured in 3D. MCF10-A cells expressing MEK2-DD contained high levels of activated Erk compared to control acini, and significantly upregulated integrin α5 expression during 3D morphogenesis; as indicated by immunoblotting of acinar structures (Fig. 7B). Overexpression of an active form of Akt (myristoylated Akt1) had no effect on integrin α5 expression (Fig. 7B). As previously shown, activation of the Mek/Erk pathway, but not the Akt pathway, blocks Bim expression in 3D acini (Fig. 7B) (Reginato et al., 2005). These results reveal that the Mek/Erk pathway in ErbB2 expressing 3D structures regulates integrin α5 expression.

Taken together, these results indicate that increased levels of integrin α5 and Src activity in suspension of mammary epithelial cells functions as a survival mechanism to transiently protect cells against anokis. However, in cells overexpressing RTKs, such as ErbB2, elevation of integrin α5 and Src activity can maintain RTKs active and allow Erk activation and inhibition of Bim expression, and

chronically block anoikis. Additionally, this data suggests that RTKs, such as ErbB2, are dependent on specific integrin subunits for anoikis resistance.

DISCUSSION

Our findings show overexpressed growth factor receptors require specific integrin subunits for anoikis resistance at the single cell level, but also in the more tissue-relevant context of 3D culture. Previous work using normal epithelial cells, showed that integrin-mediated adhesion was required for proper growth factor survival signaling (Reginato et al., 2003). Overexpression of growth factor receptors, which is found in many epithelial carcinomas, is thought to uncouple and bypass the requirement for integrin signaling in the absence of adhesion. Our results here reveal that growth factor receptors can regulate specific integrin subunits to maintain their own activation and thus remain highly dependent on integrin function under conditions of no or reduced matrix adhesion. We show that epithelial cells overexpressing ErbB2 upregulate integrin α5 RNA and protein and that integrin α5 is required for ErbB2-mediated anoikis resistance associated with luminal filling during morphogenesis of spheroid epithelial structures in vitro. Moreover, integrin α5 is required in maintaining elevated c-Src activity contributing to ErbB2 phosphorylation and downstream Mek/Erk-mediated inhibition of Bim during loss of adhesion. Activation of c-Src by cells in suspension is required for ErbB2 phosphorylation and ErbB2-mediated Bim inhibition and anoikis resistance. In addition, we show that normal epithelial cells also elevate expression of integrin α5 when detached from matrix allowing activation of c-Src in suspension to delay anoikis possibly by maintaining RTK signaling such as EGF receptor. However, EGFR expression is not maintained in normal cells without adhesion (Reginato et al., 2003). Under conditions when RTKs, such as ErbB2, are overexpressed, our data suggests that c-Src activation during matrix deprivation functions to maintain RTKs active and protect from anoikis. Indeed, recent data in prostate cancer cells show that c-Src activation during cell detachment

phosphorylates EGF receptor on pY845 (site homologues to ErbB2 p877) during cell detachment and blocks anoikis via Erk/Bim axis (Giannoni et al., 2009). The integrin α5/c-Src survival pathway may be specific for RTKs since reducing integrin α5 had no effect on anchorage independent growth of Ras overexpressing cells. Thus, elevating integrin α5 under low adhesion may be a general mechanism of survival from anoikis that is co-opted by RTKs in cancer cells to maintain its own activity, promote cell survival and block luminal clearing in cases where tissue architecture and cell-matrix interactions are compromised possibly in early stages of cancer.

Anoikis also serves as a barrier to metastasis (Reddig and Juliano, 2005), and since integrin α5 is known to support epithelial migration and metastasis (Imanishi et al., 2007); (Jia et al., 2004) (Murthy et al., 1996), our results suggest that integrin α5 may also contribute to survival during metastatic dissemination in the absence of matrix adhesion. Our data shows that integrin α5 expression positively correlates with ErbB2-overexpressing breast cancers. While this manuscript was under review, an article by Valastyan et al. showed that integrin α5 levels are also elevated in metastatic breast cancers compared to primary tumors and expression of integrin α5 is associated with a cohort of genes that is associated with poor clinical outcome of breast cancer patients (Valastyan et al., 2009). Indeed, consistent with our results, Valastyan et al. also showed that integrin α5 plays a critical role in anoikis resistance of breast cancer cells in vitro. Thus, integrin α5 may serve as a key player in breast cancer progression including metastasis.

Integrins and receptor tyrosine kinases are well know to collaborate and regulate a number of physiological processes including pathways that control cell growth, cell movement and survival (reviewed in, (Lee and Juliano, 2004) (Miranti and Brugge, 2002)). Loss of adhesion can negatively regulate RTKs, since detachment of normal cells can result in desensitization to EGF. For example, by inhibiting expression of EGF receptor (Reginato et al., 2003), and possibly by targeting receptors for degradation as is the case for PDGF receptor (Baron and Schwartz, 2000). Interestingly, integrin β4 has recently been shown to cooperate

with ErbB2 in a breast cancer mouse model (Guo et al., 2006). These studies showed that using a mouse model of ErbB2-induced mammary carcinoma containing an integrin β4-signaling-deficient mutant blocked tumor growth and invasion in vivo. In addition, expression of this mutant reversed ErbB2-mediated acini disorganization, disruption of cell-cell junctions, and polarity in 3D culture. In our study, reducing levels of integrin α5 had little to no effect on acinar cell growth, organization of cell-cell contacts or polarity of outer cells in direct contact with ECM. Only inner cells in 3D acini not in direct contact with ECM became sensitized to loss of integrin α5. This is consistent with our finding that reducing integrin α5 in ErbB2-cells cultured in 2D did not alter cell growth or induce apoptosis in cells attached to plastic, but only had an effect during cell detachment. Our study suggests that ErbB2 activity and survival function becomes highly dependent on integrin α5 in the absence of strong ECM adhesions, and that ErbB2 may depend on other integrins, such as α6/β4, for amplifying signals when in direct contact with ECM.

Evidence of adhesion-independent, growth factor-dependent activities of integrins is also emerging and may be especially relevant in cancer cells overexpressing RTKs (Comoglio et al., 2003). Tumor cells overexpressing RTKs may be able to uncouple from normal regulation by cell adhesion, and also utilize integrins as adaptors to amplify signals. For example, in various tumor cell lines integrin β4 can act as a functional signaling molecule without engagement of its extracellular ligand-binding domain by complexing with the hepatocyte growth factor (HGF) receptor Met (Trusolino et al., 2001). Integrin β4 conspires with Met receptor for anchorage–independent growth by activating Met signaling towards the Ras-Erk cascade (Bertotti et al., 2006). It is still also possible that matrix adhesions do contribute to the integrin α5 regulation of ErbB2 during cell detachment, as it has been shown for the autocrine loop of laminin-5-α6β4-Rac described in the HMT-3544 human breast cancer progression series (Zahir et al., 2003). However, we did not observe changes in FAK activity in ErbB2 overexpressing cells compared to normal cells, and did not detect any FAK activity in suspension, suggesting that FAK-dependent adhesion

signaling was not involved. In addition, we were unable to detect any fibronectin, the major ligand for integrin α5, staining in centrally located cells in acini overexpressing ErbB2 (data not shown). Moreover, studies using mammary epithelial cells overexpressing ErbB2 treated with integrin β1 and α5 blocking antibodies had no effect on colony formation in soft agar, suggesting that matrix-derived signals may not be required for ErbB2-mediated anchorage independence (Jenndahl et al., 2006). Whether integrin α5 has adhesion-independent adaptor functions for ErbB2 activation, as has been shown for integrin β4 regulation of the Met tyrosine kinase (Trusolino et al., 2001), remains to be determined.

Activation of c-Src is known to play a significant role in integrin and receptor tyrosine kinase cross talk, allowing synergy between these pathways, especially in transformed cells (Bromann et al., 2004). Our work shows that integrin α5 allows maintenance of Src phosphorylation in the absence of cell adhesion that in turn allows ErbB2 activation and survival from anoikis. A number of studies have shown that detachment from the substratum induces an increase in the phosphorylation and activity of c-Src in tumor cell lines (Wei et al., 2004) and in nonmalignant rat intestinal epithelial cells (Loza-Coll et al., 2005). Intriguingly, in rat intestinal cells c-Src activity is transiently increased during cell detachment and contributes to transient protection from anoikis for about two hours. Moreover, Src activation in suspension correlates with anoikis resistance, as six anoikis-resistant lung cancer cell lines were found to induce and maintain c-Src activation upon cell detachment (Wei et al., 2004). Our studies suggest that overexpression of RTKs, such as ErbB2, induce expression of specific integrins to help maintain c-Src activity in suspension. It is possible that elevated levels of integrin α5 help recruit c-Src to the plasma membrane, in the absence of adhesion, where it can proceed to activate ErbB2 on Tyr-877 and maintain ErbB2 activity therefore inhibiting Bim expression and suppressing anoikis. Recent data in prostate cancer cells suggest a similar model of Src regulation of EGF receptor activation during cell detachment that maintains Erk active in suspension and blocks Bim expression and anoikis (Giannoni et al., 2009). Although it is not clear how c-

Src becomes active during cell detachment, studies have shown that some tyrosine kinase receptors, such as EGFR, and PDGFR, can bind to c-Src via its SH2 domain and activate it (Bromann et al., 2004). However, we did not detect major differences in c-Src activity in cells overexpressing ErbB2 compared to parental MCF-10A cells (data not shown), suggesting that ErbB2 may not be directly involved in regulating c-Src activity in absence of adhesion. Recent studies showed that maintenance of c-Src activation in suspended prostate cancer cells required production of reactive oxygen species (ROS) (Giannoni et al., 2009). It is not clear whether ROS plays a role in c-Src regulation during anoikis in MCF-10A cells. However, it will be of interest to determine whether integrin α5 plays a role in ROS regulation of c-Src during cell detachment.

Accumulating evidence suggests that overexpression or increased activity of c-Src plays a key role in ErbB2-mediated anchorage independence and cell survival (Bromann et al., 2004; Ishizawar and Parsons, 2004). However, the link to apoptotic pathways has not been well characterized. Previous studies have implicated c-Src regulation of Stat-3 control of Bcl-xL in cells expressing EGFR and ErbB2 (Karni et al., 1999; Song et al., 2003), but in our studies we did not detect changes in Bcl-xL expression in ErbB2 overexpressing cells when compared to control MCF-10A cells or in ErbB2 cells targeted with integrin α5 RNAi (data not shown). Our finding that c-Src is required for ErbB2-mediated inhibition of Bim during anoikis may explain in part how c-Src regulates ErbB2-mediated anchorage-independent survival. Interestingly, non-small cell lung cancer cell (NSCLC) lines dependent on EGF receptor for survival contain elevated phosphorylation of c-Src-family kinase. Treatment of these cells with c-Src inhibitor reduced phosphorylation of EGFR and ErbB2, and induced apoptosis (Zhang et al., 2007). The regulation of Bcl-2 family members was not examined in this study, but it is likely that induction of Bim may play a key role, since a number of groups have implicated regulation of Bim as essential for apoptosis induced by a range of EGFR/ErbB2 inhibitors in NSCLC (Costa et al., 2007; Cragg et al., 2007; de La Motte Rouge et al., 2007; Deng et al., 2007) and breast cancer cell lines (Piechocki et al., 2007). Thus, our studies suggest that the

integrin α5 and c-Src regulation of ErbB2-Erk axis is not only critical for regulating Bim, anoikis resistance, and tumor oncogenesis, but may also be a vital cascade for developing novel strategies for cancer therapy.

MATERIAL AND METHODS

Cell culture and materials.

MCF-10A cells were obtained from the American Type Culture Collection and maintained as described previously (Reginato et al., 2003). Briefly, cells were cultured in DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 5% donor horse serum, 20 ng/ml EGF (Peprotech, Rocky Hill, NJ), 10 μg/ml insulin (Sigma, St Louis, MO), 1 ng/ml cholera toxin (Sigma), 100 μg/ml hydrocortisone (Sigma), 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). Poly-HEMA and methylcellulose were purchased from Sigma. LY294002 and U0126 were purchased from Calbiochem (San Diego, CA). PP1 and PP2 were purchased from Biomol (Plymouth Meeting, PA). Growth factor-reduced Matrigel was obtained from BD Biosciences (San Diego, CA). Primary antibodies to phospho-ErbB2/Tyr 877, α-Cleaved Caspase 3, phospho-Mek1/2 (Ser217/221), phospho-Akt (Ser473), Akt, phospho-Src (Tyr416), Src were obtained from Cell Signaling (Danvers, MA). Antibodies for Actin, Integrin α5, Mek1, IgG-HRP and Erk2 were obtained from Santa Cruz Biotech (Santa Cruz, CA). Antibodies for integrin α5 (for immunofluorescence), integrin β1 and phosphotyrosine-PY20 were obtained from BD Biosciences (San Diego, CA). Antibodies for integrin α3 and integrin α6 were obtained from Chemicon (Temecula, CA). Antibodies for ErbB2 (Calbiochem, EMD Chemicals, La Jolla, CA), phospho-Erk1/2 [$pTpY^{185/187}$] (Biosource, Camarillo, CA), and Bim (Stressgen, Ann Arbor, Michigan) were also obtained.

Retrovirus vectors and stable cell lines

Retroviral vectors pBabe-NeuN, (human wildtype ErbB2), and pBabe-MEK2-DD for stable gene expression have been described previously (Gunawardane et al., 2005; Reginato et al., 2003). Constitutively active ErbB2 mutant (pBabe-NeuT) was kindly provided by Danielle Carroll (Harvard Medical School, Boston, MA). pLNCX-Myr-Akt1 was kindly provided by P. Tsichlis (Tufts-New England Medical Center, Boston, MA). Vesicular stomatitis virus G protein-pseudotyped retroviruses were produced and MCF-10A cells were infected and selected as previously described (Reginato et al., 2003).

Quantitative real time PCR (qRT-PCR)

Total RNA was isolated from cells using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen). Equal amounts of total RNA (250ng) were added to Brilliant II QRT-PCR master mix (Stratagene, La Jolla, CA) with primer/probe sets purchased from Applied Biosystems (Foster City, CA). PCR reactions were performed in a volume of 25µl using a MX 3000 machine and analysis was performed using the MxPro software according to the manufacturer's instructions (Stratagene). Gene and catalog numbers for the primer/probe sets are as follows: ITGA2 (Hs00158148_m1), ITGA5 (Hs00233732_m1), and ITGA6 (Hs01041011_m1). Expression of the housekeeping gene cyclophillin A (Hs99999904_m1) was used as an internal loading control. Reactions for each sample were performed in duplicate, then each duplicate was performed in triplicate.

Immunohistochemistry (IHC)

Tissue microarrays containing formalin-fixed paraffin-embedded breast tumor tissue samples as well as normal breast tissue samples were obtained from US Biomax, Inc (Rockville, MD). Breast cancer tissue microarrays BR961 and BRC961, containing 70 cases of HER2/ErbB2 positive tissue and 24 cases of

HER2/ErbB2 negative tissue, was de-paraffinized and rehydrated. Heat-induced antigen retrieval was performed at 95^0C for 30 minutes using the DakoCytomation Target Retrieval Solution (Dako North America, Inc, Carpinteria, CA). This was followed by a peroxide block in 3% H_2O_2 to quench endogenous peroxidase activity, and a subsequent 1 hour block in 10% goat serum in 1X Tris Buffered Saline (TBS). The tissue samples were then incubated in anti-integrin α5 antibody (Santa Cruz) at 4μg/ml in antibody diluent (DakoCytomation) overnight in a humidified chamber at room temperature. The primary antibody was detected using goat anti-rabbit IgG-peroxidase antibody (Sigma) at 35 μg/ml in antibody diluent, and signals were amplified and visualized by 3,3'-diaminobenzidine (DAB kit, DakoCytomation). Sections were counterstained using Harris hematoxylin, dehydrated and mounted using Permount solution (Fisher). For statistical analysis, all cases that stained negative (-) for integrin α5 staining were classified as negative, and all cases with +, ++ or +++ were classified as positive. Similar assessment was done for ErbB2/Her2 negative and positive tissue. For statistical analysis, Pearson's Correlation was used to analyze the relationship between integrin α5 expression and ErbB2 expression. The results were considered significant at $p<0.05$.

Western analysis

Cell lysates from 2-5 x 10^6 MCF-10A cells, either attached or in suspension for the indicated times, were prepared in RIPA lysis buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 50 mM TrisHCL at pH 8, 0.1% SDS, 10% glycerol, 5 mM EDTA, 20 mM NaF and 1 mM Na_3VO_4) supplemented with 1 μg/ml each of pepstatin, leupeptin, aprotinin, and 200 μg/ml phenyl methylsulphonyl fluoride (PMSF). Lysates were cleared by centrifugation at 16,000*g* for 20 min at 4 °C and analyzed by SDS-PAGE and autoradiography. Lysates were collected from cells in 3D as previously described (Reginato, 2005). Briefly, acini were

washed with PBS, incubated with RIPA lysis buffer for 15 min at 4^{o}C and then proteins were analyzed by western blotting using SDS-PAGE and autoradiography.

RNA interference

Transfections for siRNA were carried out as previously described (Reginato et al., 2003) (Reginato et al., 2005). Briefly, MCF-10A cells were plated onto 6-well plates at 200,000 cells/well. After 24 h, cells were transfected with double-stranded RNA−DNA hybrids at a final concentration of 1 μg annealed oligo using Oligofectamine (Invitrogen) according to manufacturer's instructions. Cells were retransfected as described at 48 h. At 76 h after initial transfection, cells were placed in specific assay at the indicated times. Initial small interfering RNA (siRNA) oligonucleotides (SMARTpool) were obtained from Dharmacon Research (Lafayette, CO) for integrin α5 and α3. Single siRNA were identified with the highest level of knockdown and purchased from Dharmacon. Control sense (luciferase) 5'- (GGCUCCCGCUGAAUUGGA AUU)d(TT)-3'; ITGA5#1 sense 5'- (GCAAGAAUCUCAACAACUCUU)d(TT)-3'; ITGA5#2 sense 5'- (GAGAGGAGCCUGUGGAGUAUU)d(TT)-3'; and ITGA3 sense 5'- (GUGUACAUCU-AUCACAGUAUU)d(TT)-3'.

Lentivirus vectors and stable cell lines

Stable cell lines for shRNA knock-downs were generated by infection with the lenti-viral vector pLKO.1-puro carrying a shRNA sequence for scrambled (Addgene (Sarbassov et al.,
2005)), Src (Sigma-Aldrich) and integrin α5 (Open Biosystems). VSVG-pseudotyped lentivirus was generated by the cotransfection of 293-T packaging cells with 10 μg of DNA and packaging vectors as described (Rubinson et al., 2003). Scrambled shRNA sequence used is CCTAAGGTTAAGTCGCCCTCGCTCTAGCGAGGGCGACTTAACCTT. Src shRNA sequence used is

CCGGGACAGACCTGTCCTTCAAGAACTCGAGTTCTTGAAGGACAGGTCTGTCTTTTTG. Integrin α5 shRNA sequence used is AGGCCATGATGAGTTTGGCCGATTCTCGAGAATCGGCC AAACTCATCATGG. MCF-10A cells overexpressing ErbB2 were infected as previously described (Reginato et al., 2003).

Colony formation in soft agar assay

Cells were grown in soft agar and fed as described (Gunawardane et al., 2005). Cells were incubated for 21 days and then stained with 500 μl of 0.05% p-Iodonitrotetrazolium (INT) – violet overnight. Colonies (>50 μM in diameter) were counted using a Leica DM IRB Inverted Research Microscope (Bannockburn, IL). All assays were conducted in duplicate in at least three independent experiments.

Detachment-induced apoptosis assay

MCF10A cells were placed in suspension in growth medium as previously described (Reginato et al., 2003). Briefly, tissue culture plates were coated with poly-HEMA (6 mg/ml), incubated at 37 °C until dry and washed with PBS before use. Cells were placed in growth medium in the presence of 0.5% methylcellulose (to avoid potential survival effects caused by clumping of cells) in suspension at a density of 500,000 cells/ml and plated on poly-HEMA-coated 60-mm plates for the indicated time. Cells were then washed with 1x PBS and counted. 25,000 cells were used to determine apoptosis with the cell death detection ELISA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. For some experiments, after cells were washed with PBS they were resuspended in 1X binding buffer from the Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen) according to the manufacturer’s instructions. FITC annexin v and propidium iodide (Annexin V-FITC kit) were then added and the cells incubated in the dark at room temperature for 15 minutes immediately followed by analysis

using Guava Technologies EasyCyte Plus, and data analyzed with Guava Cytosoft 5.3 software. In both cases, each time point was performed in duplicate and each experiment was carried out at least three times.

3D morphogenesis assay

Assays were performed as previously described (Reginato et al., 2005). Briefly, MCF-10A-ErbB2 cells were resuspended in assay medium (DMEM/F12 supplemented with 2% donor horse serum, 10 μg/ml insulin, 1 ng/ml cholera toxin, 100 μg/ml hydrocortisone, 50 U/ml penicillin, 50 μg/ml streptomycin, as well as 5 ng/ml EGF). Eight-well chamber slides (BD Falcon) were coated with 45 μl Matrigel per well and 5,000 cells were plated per well in assay medium containing final concentration of 2% matrigel. Assay medium containing 2% Matrigel was replaced every four days and chemical inhibitors, were supplemented to media as indicated.

Immunofluorescence and image acquisition.

Structures were prepared as previously described (Reginato et al., 2005). Briefly, acini were fixed in 4% formalin for 20 min. Fixed structures were washed with PBS-glycine (130 mM NaCl, 7 mM Na2HPO4, 100 mM glycine) three times for 10 minutes each time. The structures were then blocked in IF buffer (130 mM NaCl, 7 mM Na2HPO4, 3.5 mM NaH2PO4, 7.7 mM NaN3, 0.1% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween 20) plus 10% goat serum for 1 to 2 h, followed by 2^0 blocking buffer for 40 min. Primary antibodies were diluted in 2^0 blocking buffer, followed by incubation overnight at 4°C. After washing, acini were incubated with secondary antibodies coupled with Alexa fluor dyes (Molecular Probes). Structures were incubated with 0.5 ng/ml of DAPI (4', 6'-diamidino-2-phenylindole; Sigma) before being mounted with Prolong (Molecular Probes). Quantification of caspase-3 staining in ErbB2 structures was performed at x40 magnification. A minimum of 100 acinar structures were counted per

experiment, and each experiment was performed three independent times. Confocal analysis was performed using the Leica DM6000 B Confocal Microscope. Images were generated using the Leica Confocal Imaging Software System and converted to Tiff format.

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References

Aranda, V., Haire, T., Nolan, M. E., Calarco, J. P., Rosenberg, A. Z., Fawcett, J. P., Pawson, T. and Muthuswamy, S. K. (2006). Par6-aPKC uncouples ErbB2 induced disruption of polarized epithelial organization from proliferation control. *Nat Cell Biol* **8**, 1235-45.

Baron, V. and Schwartz, M. (2000). Cell adhesion regulates ubiquitin-mediated degradation of the platelet-derived growth factor receptor beta. *J Biol Chem* **275**, 39318-23.

Bertotti, A., Comoglio, P. M. and Trusolino, L. (2006). Beta4 integrin activates a Shp2-Src signaling pathway that sustains HGF-induced anchorage-independent growth. *J Cell Biol* **175**, 993-1003.

Bromann, P. A., Korkaya, H. and Courtneidge, S. A. (2004). The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene* **23**, 7957-68.

Comoglio, P. M., Boccaccio, C. and Trusolino, L. (2003). Interactions between growth factor receptors and adhesion molecules: breaking the rules. *Curr Opin Cell Biol* **15**, 565-71.

Costa, D. B., Halmos, B., Kumar, A., Schumer, S. T., Huberman, M. S., Boggon, T. J., Tenen, D. G. and Kobayashi, S. (2007). BIM mediates EGFR tyrosine kinase inhibitor-induced apoptosis in lung cancers with oncogenic EGFR mutations. *PLoS Med* **4**, 1669-79; discussion 1680.

Cragg, M. S., Kuroda, J., Puthalakath, H., Huang, D. C. and Strasser, A. (2007). Gefitinib-induced killing of NSCLC cell lines expressing mutant EGFR requires BIM and can be enhanced by BH3 mimetics. *PLoS Med* **4**, 1681-89; discussion 1690.

de La Motte Rouge, T., Galluzzi, L., Olaussen, K. A., Zermati, Y., Tasdemir, E., Robert, T., Ripoche, H., Lazar, V., Dessen, P., Harper, F. et al. (2007). A novel epidermal growth factor receptor inhibitor promotes apoptosis in non-small cell lung cancer cells resistant to erlotinib. *Cancer Res* **67**, 6253-62.

Debnath, J. and Brugge, J. S. (2005). Modelling glandular epithelial cancers in three-dimensional cultures. *Nat Rev Cancer* **5**, 675-88.

Debnath, J., Mills, K. R., Collins, N. L., Reginato, M. J., Muthuswamy, S. K. and Brugge, J. S. (2002). The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. *Cell* **111**, 29-40.

Deng, J., Shimamura, T., Perera, S., Carlson, N. E., Cai, D., Shapiro, G. I., Wong, K. K. and Letai, A. (2007). Proapoptotic BH3-only BCL-2 family protein BIM connects death signaling from epidermal growth factor receptor inhibition to the mitochondrion. *Cancer Res* **67**, 11867-75.

Frisch, S. M. and Screaton, R. A. (2001). Anoikis mechanisms. *Curr Opin Cell Biol* **13**, 555-62.

Giannoni, E., Fiaschi, T., Ramponi, G. and Chiarugi, P. (2009). Redox regulation of anoikis resistance of metastatic prostate cancer cells: key role for Src and EGFR-mediated pro-survival signals. *Oncogene* **28**, 2074-86.

Gunawardane, R. N., Sgroi, D. C., Wrobel, C. N., Koh, E., Daley, G. Q. and Brugge, J. S. (2005). Novel role for PDEF in epithelial cell migration and invasion. *Cancer Res* **65**, 11572-80.

Guo, W., Pylayeva, Y., Pepe, A., Yoshioka, T., Muller, W. J., Inghirami, G. and Giancotti, F. G. (2006). Beta 4 integrin amplifies ErbB2 signaling to promote mammary tumorigenesis. *Cell* **126**, 489-502.

Harris, J. R. (2004). Diseases of the breast. Philadelphia: Lippincott Williams & Wilkins.

Hynes, N. E. and Stern, D. F. (1994). The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochim Biophys Acta* **1198**, 165-84.

Imanishi, Y., Hu, B., Jarzynka, M. J., Guo, P., Elishaev, E., Bar-Joseph, I. and Cheng, S. Y. (2007). Angiopoietin-2 stimulates breast cancer metastasis through the alpha(5)beta(1) integrin-mediated pathway. *Cancer Res* **67**, 4254-63.

Ishizawar, R. and Parsons, S. J. (2004). c-Src and cooperating partners in human cancer. *Cancer Cell* **6**, 209-14.

Ishizawar, R. C., Miyake, T. and Parsons, S. J. (2007). c-Src modulates ErbB2 and ErbB3 heterocomplex formation and function. *Oncogene* **26**, 3503-10.

Jenndahl, L. E., Taylor-Papadimitriou, J. and Baeckstrom, D. (2006). Characterization of integrin and anchorage dependence in mammary epithelial cells following c-erbB2-induced epithelial-mesenchymal transition. *Tumour Biol* **27**, 50-8.

Jia, Y., Zeng, Z. Z., Markwart, S. M., Rockwood, K. F., Ignatoski, K. M., Ethier, S. P. and Livant, D. L. (2004). Integrin fibronectin receptors in matrix metalloproteinase-1-dependent invasion by breast cancer and mammary epithelial cells. *Cancer Res* **64**, 8674-81.

Karni, R., Jove, R. and Levitzki, A. (1999). Inhibition of pp60c-Src reduces Bcl-XL expression and reverses the transformed phenotype of cells overexpressing EGF and HER-2 receptors. *Oncogene* **18**, 4654-62.

Lee, J. W. and Juliano, R. (2004). Mitogenic signal transduction by integrin- and growth factor receptor-mediated pathways. *Mol Cells* **17**, 188-202.

Lohrisch, C. and Piccart, M. (2001). An overview of HER2. *Semin Oncol* **28**, 3-11.

Loza-Coll, M. A., Perera, S., Shi, W. and Filmus, J. (2005). A transient increase in the activity of Src-family kinases induced by cell detachment delays anoikis of intestinal epithelial cells. *Oncogene* **24**, 1727-37.

Mailleux, A. A., Overholtzer, M., Schmelzle, T., Bouillet, P., Strasser, A. and Brugge, J. S. (2007). BIM regulates apoptosis during mammary ductal morphogenesis, and its absence reveals alternative cell death mechanisms. *Dev Cell* **12**, 221-34.

Miranti, C. K. and Brugge, J. S. (2002). Sensing the environment: a historical perspective on integrin signal transduction. *Nat Cell Biol* **4**, E83-90.

Murthy, M. S., Reid, S. E., Jr., Yang, X. F. and Scanlon, E. P. (1996). The potential role of integrin receptor subunits in the formation of local recurrence and distant metastasis by mouse breast cancer cells. *J Surg Oncol* **63**, 77-86.

Muthuswamy, S. K., Li, D., Lelievre, S., Bissell, M. J. and Brugge, J. S. (2001). ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nat Cell Biol* **3**, 785-92.

Nahta, R., Yu, D., Hung, M. C., Hortobagyi, G. N. and Esteva, F. J. (2006). Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. *Nat Clin Pract Oncol* **3**, 269-80.

Piechocki, M. P., Yoo, G. H., Dibbley, S. K. and Lonardo, F. (2007). Breast cancer expressing the activated HER2/neu is sensitive to gefitinib in vitro and in vivo and acquires resistance through a novel point mutation in the HER2/neu. *Cancer Res* **67**, 6825-43.

Reddig, P. J. and Juliano, R. L. (2005). Clinging to life: cell to matrix adhesion and cell survival. *Cancer Metastasis Rev* **24**, 425-39.

Reginato, M. J., Mills, K. R., Becker, E. B., Lynch, D. K., Bonni, A., Muthuswamy, S. K. and Brugge, J. S. (2005). Bim regulation of lumen formation in cultured mammary epithelial acini is targeted by oncogenes. *Mol Cell Biol* **25**, 4591-601.

Reginato, M. J., Mills, K. R., Paulus, J. K., Lynch, D. K., Sgroi, D. C., Debnath, J., Muthuswamy, S. K. and Brugge, J. S. (2003). Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. *Nat Cell Biol* **5**, 733-40.

Reginato, M. J. and Muthuswamy, S. K. (2006). Illuminating the center: mechanisms regulating lumen formation and maintenance in mammary morphogenesis. *J Mammary Gland Biol Neoplasia* **11**, 205-11.

Schwartz, M. A. (1997). Integrins, oncogenes, and anchorage independence. *J Cell Biol* **139**, 575-8.

Simpson, C. D., Anyiwe, K. and Schimmer, A. D. (2008). Anoikis resistance and tumor metastasis. *Cancer Lett* **272**, 177-85.

Song, L., Turkson, J., Karras, J. G., Jove, R. and Haura, E. B. (2003). Activation of Stat3 by receptor tyrosine kinases and cytokines regulates survival in human non-small cell carcinoma cells. *Oncogene* **22**, 4150-65.

Trusolino, L., Bertotti, A. and Comoglio, P. M. (2001). A signaling adapter function for alpha6beta4 integrin in the control of HGF-dependent invasive growth. *Cell* **107**, 643-54.

Valastyan, S., Reinhardt, F., Benaich, N., Calogrias, D., Szasz, A. M., Wang, Z. C., Brock, J. E., Richardson, A. L. and Weinberg, R. A. (2009). A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis. *Cell* **137**, 1032-46.

Wei, L., Yang, Y., Zhang, X. and Yu, Q. (2004). Altered regulation of Src upon cell detachment protects human lung adenocarcinoma cells from anoikis. *Oncogene* **23**, 9052-61.

White, D. E., Kurpios, N. A., Zuo, D., Hassell, J. A., Blaess, S., Mueller, U. and Muller, W. J. (2004). Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. *Cancer Cell* **6**, 159-70.

Xu, W., Yuan, X., Beebe, K., Xiang, Z. and Neckers, L. (2007). Loss of Hsp90 association up-regulates Src-dependent ErbB2 activity. *Mol Cell Biol* **27**, 220-8.

Zahir, N., Lakins, J. N., Russell, A., Ming, W., Chatterjee, C., Rozenberg, G. I., Marinkovich, M. P. and Weaver, V. M. (2003). Autocrine laminin-5 ligates alpha6beta4 integrin and activates RAC and NFkappaB to mediate anchorage-independent survival of mammary tumors. *J Cell Biol* **163**, 1397-407.

Zhang, J., Kalyankrishna, S., Wislez, M., Thilaganathan, N., Saigal, B., Wei, W., Ma, L., Wistuba, II, Johnson, F. M. and Kurie, J. M. (2007). SRC-family kinases are activated in non-small cell lung cancer and promote the survival of epidermal growth factor receptor-dependent cell lines. *Am J Pathol* **170**, 366-76.

Figure Legends

Fig. 1. Integrin α5 expression is upregulated by ErbB2 overexpression cells. (A) RNA was prepared from attached MCF-10A cells infected with retrovirus of empty vector (pBabe), wild-type ErbB2 (NeuN), or constitutively active ErbB2 (NeuT). Quantitative RT-PCR was used to compare mRNA levels for integrin α2, integrin α5 and integrin α6. The values were normalized to cyclophilin expression, and are represented as mean ± SE of the fold change for at least 3 individual experiments (**$p<0.05$, ***$p< 0.01$ by Students *t* test). (B) Lysates from MCF-10A cells expressing pBabe empty vector, NeuN or NeuT were analyzed by Western analysis with indicated antibodies. (C) MCF-10A cells expressing empty vector or wild-type ErbB2 (NeuN) were placed in morphogenesis assays and cell lysates collected at indicated times. Protein levels were analyzed by immunoblotting with indicated. (D) Cells expressing empty vector or wild-type ErbB2 (NeuN) were placed in morphogenesis assays and were fixed and immunostained at day 8 with antibodies for anti-integrin α5 (green) and integrin α6 (red), and nuclei were counterstained with DAPI (blue).

Fig. 2 Integrin α5 is elevated in ErbB2 positive breast cancers. (A) Normal and tumor tissue from human breast tissue microarrays were immunohistochemically stained with an anti-integrin α5 antibody (brown) and counterstained with hematoxylin (blue) and the expression of integrin α5 was analyzed. Representative images from 6 tissue sections are shown (magnification x20). (B) Immunohistochemistry

was used to determine integrin α5 expression, which was classified as described in the Methods section. From 94 cases, 64 out of 70 ErbB2-positive cases were also found to stain positive for integrin α5. A two-tailed Pearson's correlation test was used to analyze the relationship between integrin α5 expression and ErbB2/Her2 status. Results were considered to be significant at $p<0.05$.

Fig. 3. Integrin α5 expression is required for ErbB2-mediated inhibition of luminal apoptosis during morphogenesis. (A) At 48 hours after initial transfection with siRNA oligonucleotides against integrin α5 or luciferase control, MCF-10A cells stably overexpressing wildtype ErbB2 (NeuN) were placed in 3D morphogenesis assay for the indicated times. Lysates were then prepared and analyzed by Western blots using indicated antibodies. (B) Transfected ErbB2 (NeuN) cells were cultured in 3D morphogenesis assay for 10 or 12 days and then fixed and stained with the activated (cleaved) caspase-3 antibody (red, apoptosis marker). Nuclei were counterstained with DAPI (blue). (C) Acini positive for activated (cleaved) caspase-3 were counted and the fold change in caspase-3 positivity per experiment was plotted. Acini containing two or more activated caspase-3 cells were scored as positive. ($n = 3$, > 100 acini/experiment; mean ± s.e.m. of the fold change. **$p<0.05$, ***$p< 0.01$ by Students *t* test).

Fig. 4. Integrin α5 is required for ErbB2-mediated anoikis resistance, Bim inhibition, and anchorage independence. (A) At 48 hours after initial transfection with siRNA oligonucleotides against integrin α5 or luciferase control, MCF-10A cells stably overexpressing wildtype ErbB2 (NeuN) were harvested as attached cells, or placed in suspension for 48 hours and then analyzed for apoptosis using DNA Fragmentation ELISA. Values represent the mean ± s.e.m. of the fold change of A_{405} for at least three independent experiments (**$p<0.05$ by Students *t* test when comparing results for the 48h suspension cells). (B) In parallel experiments, transfected cells were lysed from monolayer culture or from 48hr

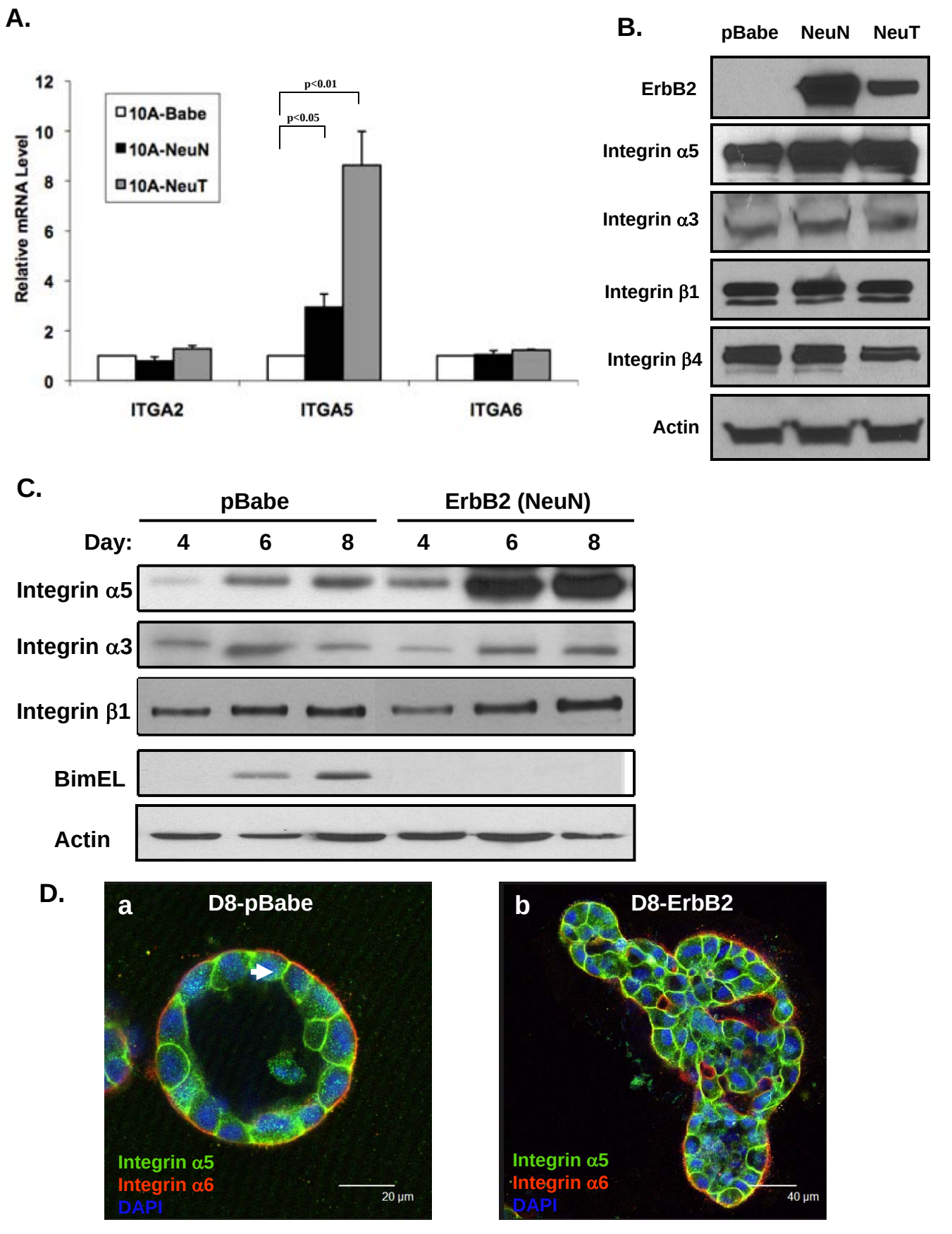
suspension culture and proteins analyzed by immunoblotting with indicated antibodies. (C) MCF-10A cells stably overexpressing activated ErbB2 (NeuT) were transfected with luciferase control or integrin α5 siRNA oligos. At 48 hours after initial transfection cells were placed in soft agar plates for 21 days and then stained with INT violet. The number of soft agar colonies was determined and the percentages normalized to that of control siRNA. The values shown represent averages of normalized values (n = 3, mean ± s.e.m. (**p<0.001, ***p<0.05 by Students *t* test)).

Fig. 5. Integrin α5 is required for ErbB2-mediated signaling. (A) MCF-10A cells stably overexpressing wildtype ErbB2 (pBabe-NeuN) were transfected with luciferase control or integrin α5 siRNA oligos. At 48 hours after initial transfection cells were placed in suspension for indicated times before lysis. Proteins were analyzed by immunoblotting with indicated. (B) MCF-10A ErbB2 (pBabe-NeuN) cells were transfected with luciferase control or integrin α5 siRNA oligos. At 48 hours after initial transfection cells were placed in suspension for indicated times before lysis. Proteins were analyzed by immunoblotting with indicated antibodies.

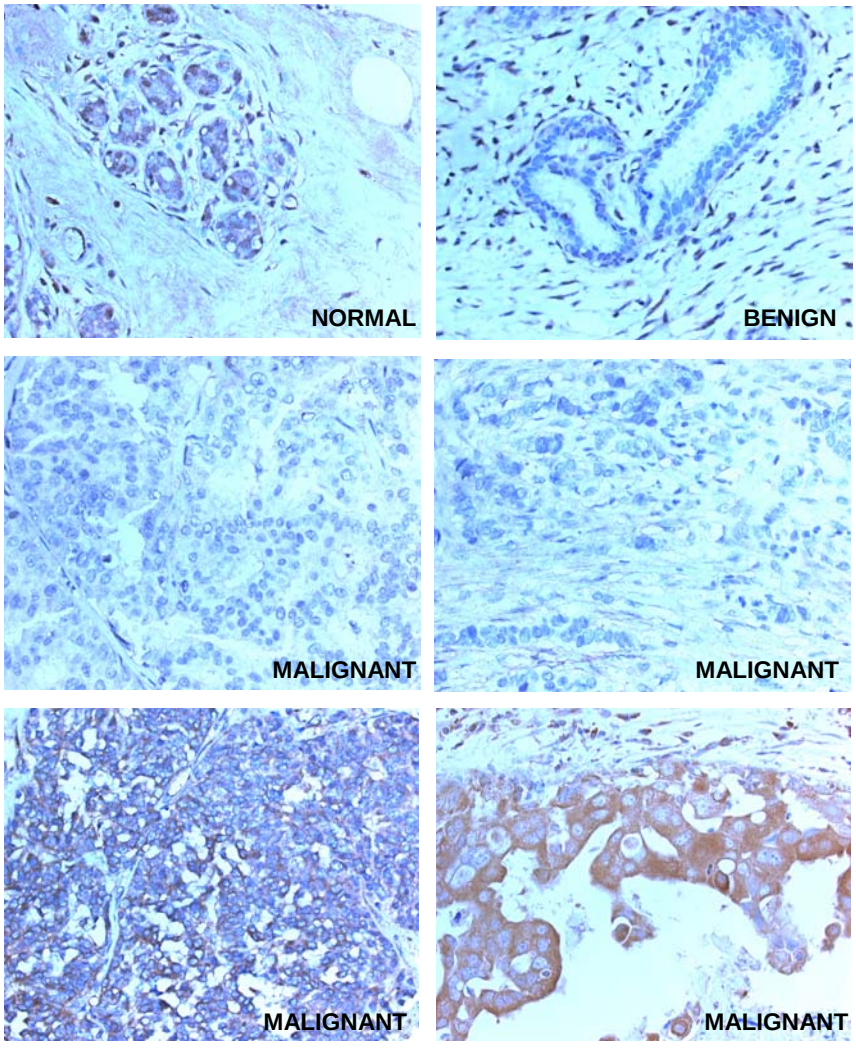
Fig. 6. Integrin regulation of ErbB2-mediated survival is associated with c-Src activation in suspension and morphogenesis. (A-C) MCF-10A cells stably overexpressing NeuN were cultured in 3D morphogenesis assay and treated on day 8 with DMSO vehicle control or PP2 (10μM). (A) At days 10 and 12, acini were fixed and stained with antibody against activated caspase-3 (red). Nuclei were counterstained with DAPI (blue). Representative confocal images for day 12 are shown, and (B) acini positive for activated caspase-3 were counted at Day 10 and Day 12 following treatment and the percent of the total acini are plotted (n = 3, mean ± s.d., > 100 acini/experiment (**p<0.001, ***p<0.0001 by Students *t* test)). (C) Cell lysates were prepared on Day 12 and proteins were analyzed by immunoblotting with indicated antibodies.

(D) MCF-10A-NeuN cells were infected with lentivirus carrying shRNA constructs against c-Src or scrambled sequence control. Cells were lysed from cells after 48hr suspension culture, and proteins analyzed by immunoblotting with indicated antibodies. (E) MCF-10A-NeuN cells infected with lentivirus carrying shRNA against c-Src or scrambled sequence control were placed in suspension for 48 hours. Cells were then stained with annexin V and propidium iodide, followed by FACS analysis (n=3). Black bars represent annexin V stain (early apoptosis), gray bars represent propidium iodide stain (late apoptosis). Histograms are plotted as mean ± s.e.m, *$p<0.05$ by Students *t* test.

Fig. 7. ErbB2 regulates integrin α5 expression via the Mek/Erk pathway during morphogenesis. (A) MCF-10A ErbB2 (pBabe-NeuN) cells were cultured in 3D morphogenesis assay and treated on day 4 with DMSO vehicle control, U0126 (10μM) or LY294002 (50μM). At day 6, acini were lysed and proteins analyzed by immunoblotting with indicated antibodies. (B) MCF-10A stably overexpressing Mek2-DD, Myr-Akt or pBabe control vector were cultured in 3D morphogenesis assays and lysed at day 4, day 6 or day 8. Proteins were analyzed by immunoblotting with indicated antibodies.


A.
Relative mRNA Level
12
10
8
6
4
2
0
10A-Babe
10A-NeuN
10A-NeuT
p<0.01
p<0.05
ITGA2
ITGA5
ITGA6
B.
pBabe
NeuN
NeuT
ErbB2
Integrin α5
Integrin α3
Integrin β1
Integrin β4
Actin
C.
pBabe
ErbB2 (NeuN)
Day:
4
6
8
4
6
8
Integrin α5
Integrin α3
Integrin β1
BimEL
Actin
D.
a
D8-pBabe
Integrin α5
Integrin α6
DAPI
20 μm
b
D8-ErbB2
Integrin α5
Integrin α6
DAPI
40 μm

A.



ErbB2 negative
ITGA5 negative

ErbB2 negative
ITGA5 negative

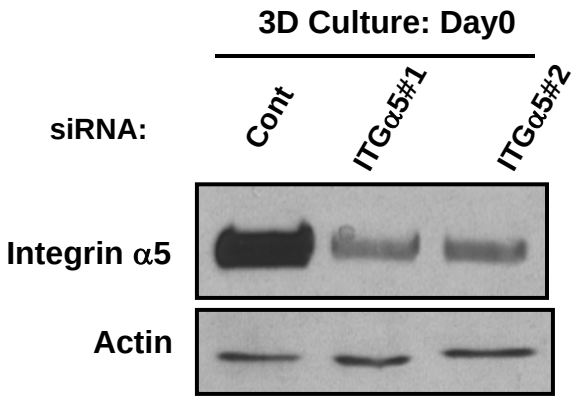
ErbB2 positive
ITGA5 positive

B.

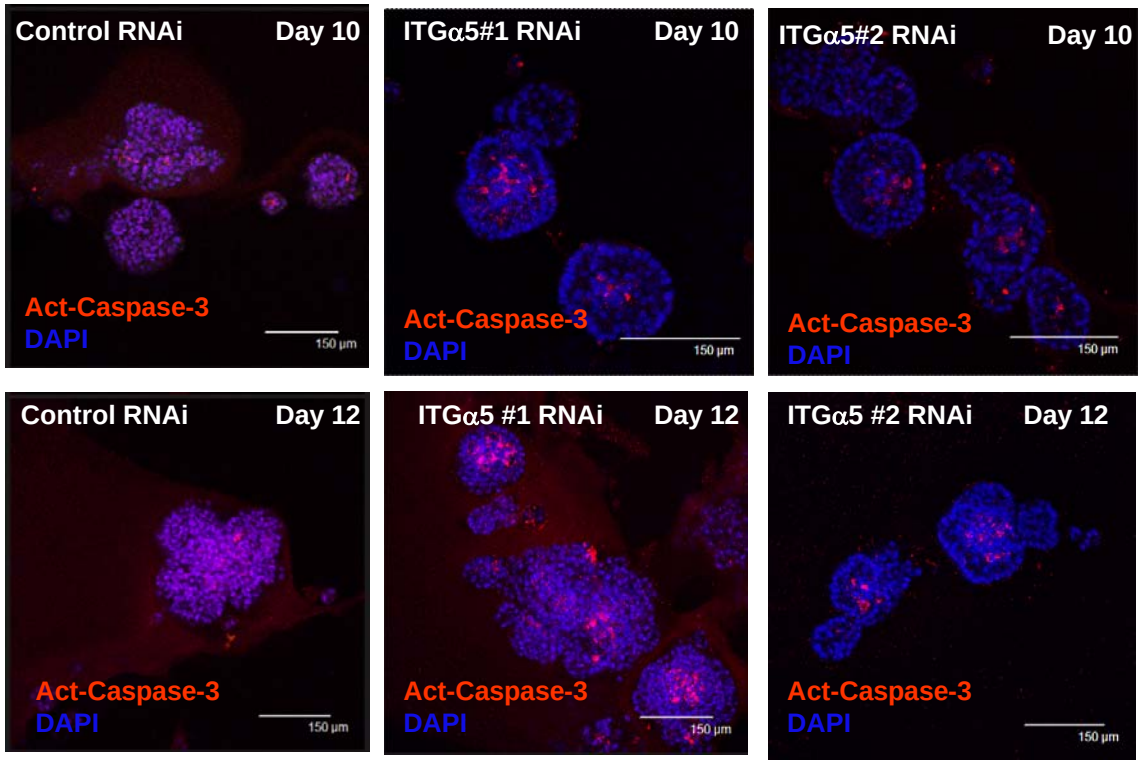
Relationship between Integrin α5 and ErbB2	Value
Pearson Correlation Score	0.219*
Significance (2-tailed)	0.034
Sample Number	94

*Correlation is significant at the 0.05 level for a 2-tailed distribution

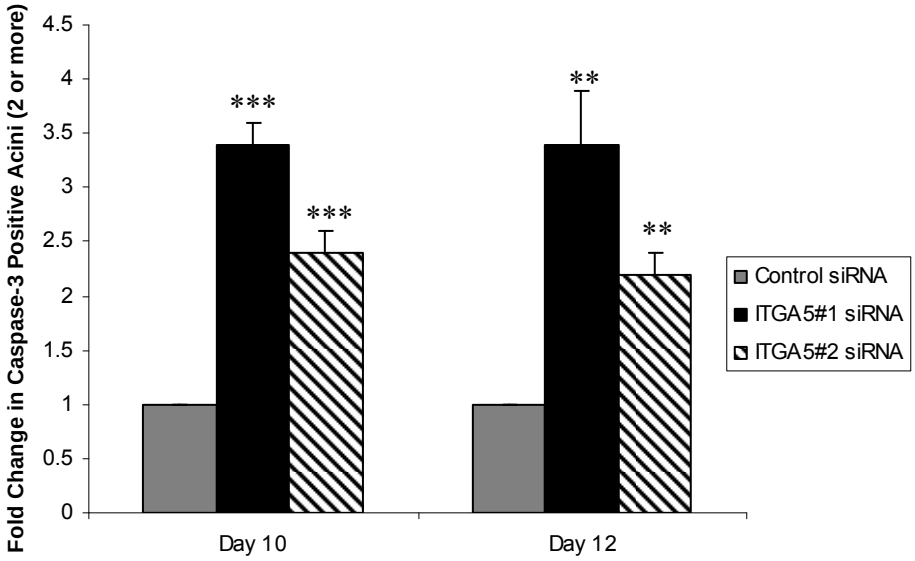
A.

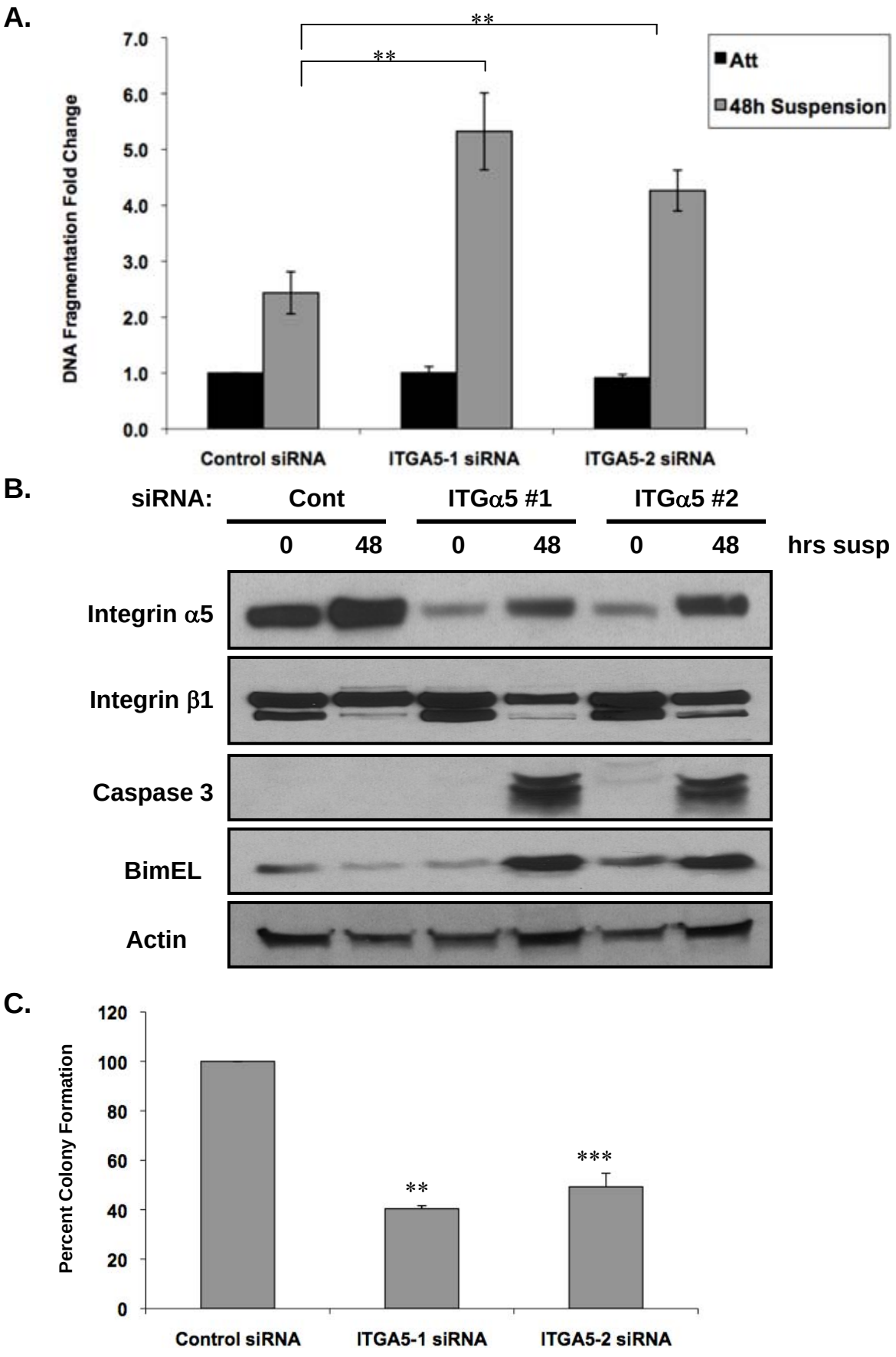

3D Culture: Day0
siRNA:
Cont
ITGα5#1
ITGα5#2
Integrin α5
Actin

B.

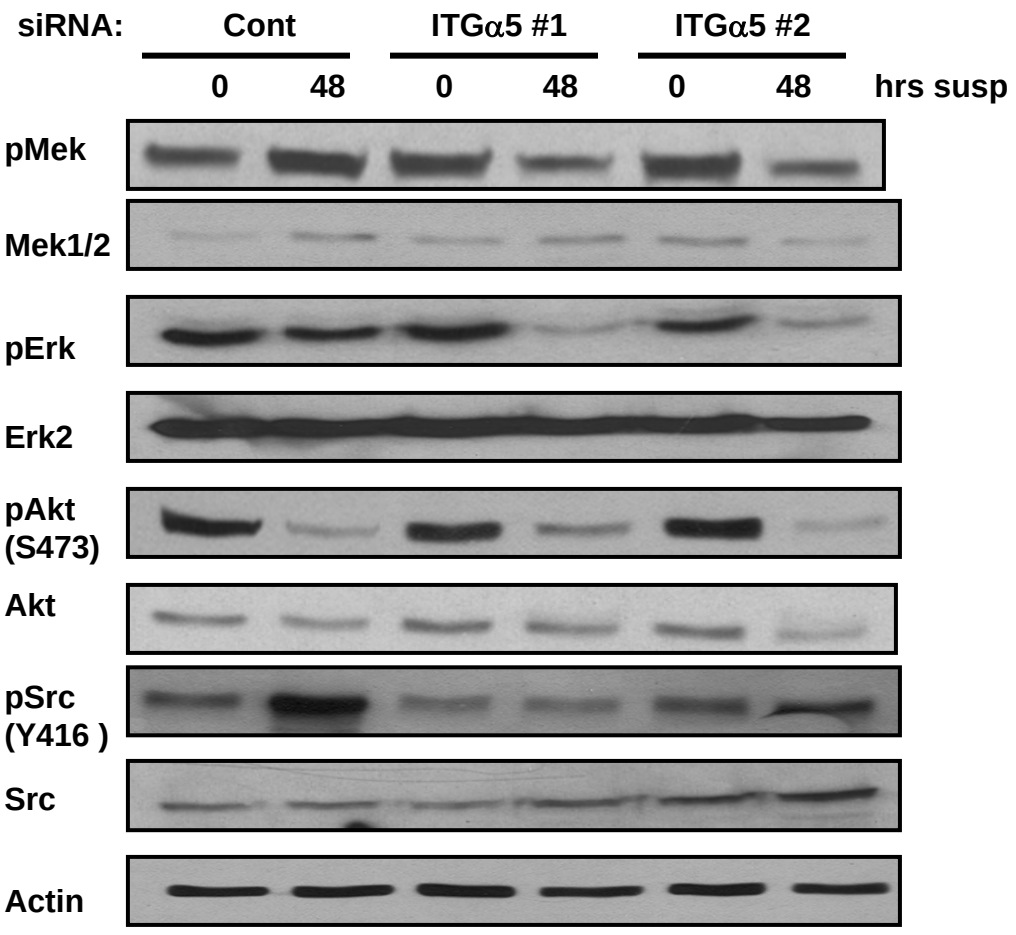

Control RNAi Day 10
ITGα5#1 RNAi Day 10
ITGα5#2 RNAi Day 10
Control RNAi Day 12
ITGα5 #1 RNAi Day 12
ITGα5 #2 RNAi Day 12
Act-Caspase-3
DAPI
150 μm

C.

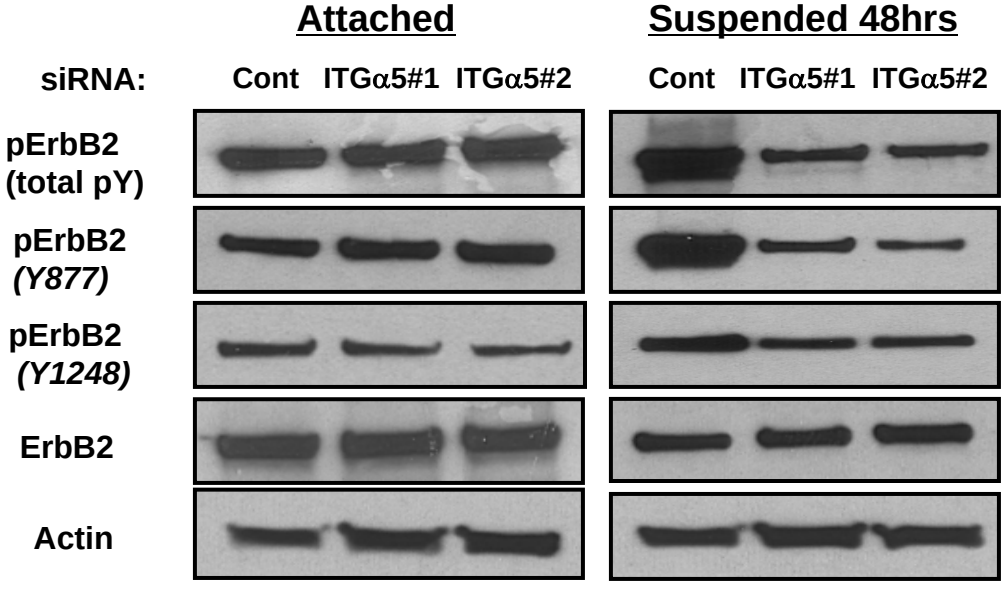

Fold Change in Caspase-3 Positive Acini (2 or more)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
***
***
**
**
Day 10
Day 12
Control siRNA
ITGA5#1 siRNA
ITGA5#2 siRNA

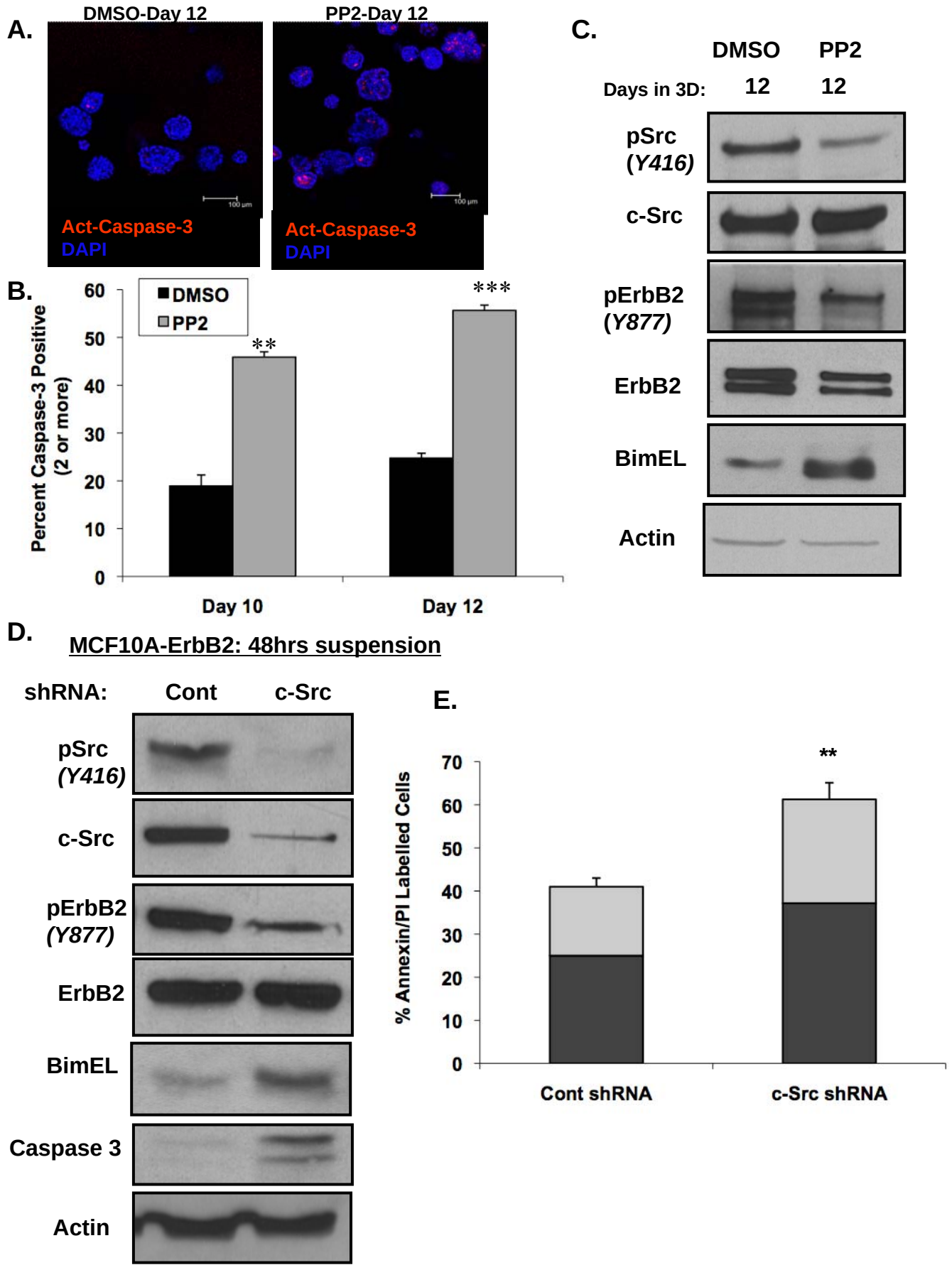

A.
DNA Fragmentation Fold Change
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
**
**
Att
48h Suspension
Control siRNA
ITGA5-1 siRNA
ITGA5-2 siRNA
B.
siRNA:
Cont
ITGα5 #1
ITGα5 #2
0
48
0
48
0
48
hrs susp
Integrin α5
Integrin β1
Caspase 3
BimEL
Actin
C.
Percent Colony Formation
120
100
80
60
40
20
0
**
***
Control siRNA
ITGA5-1 siRNA
ITGA5-2 siRNA

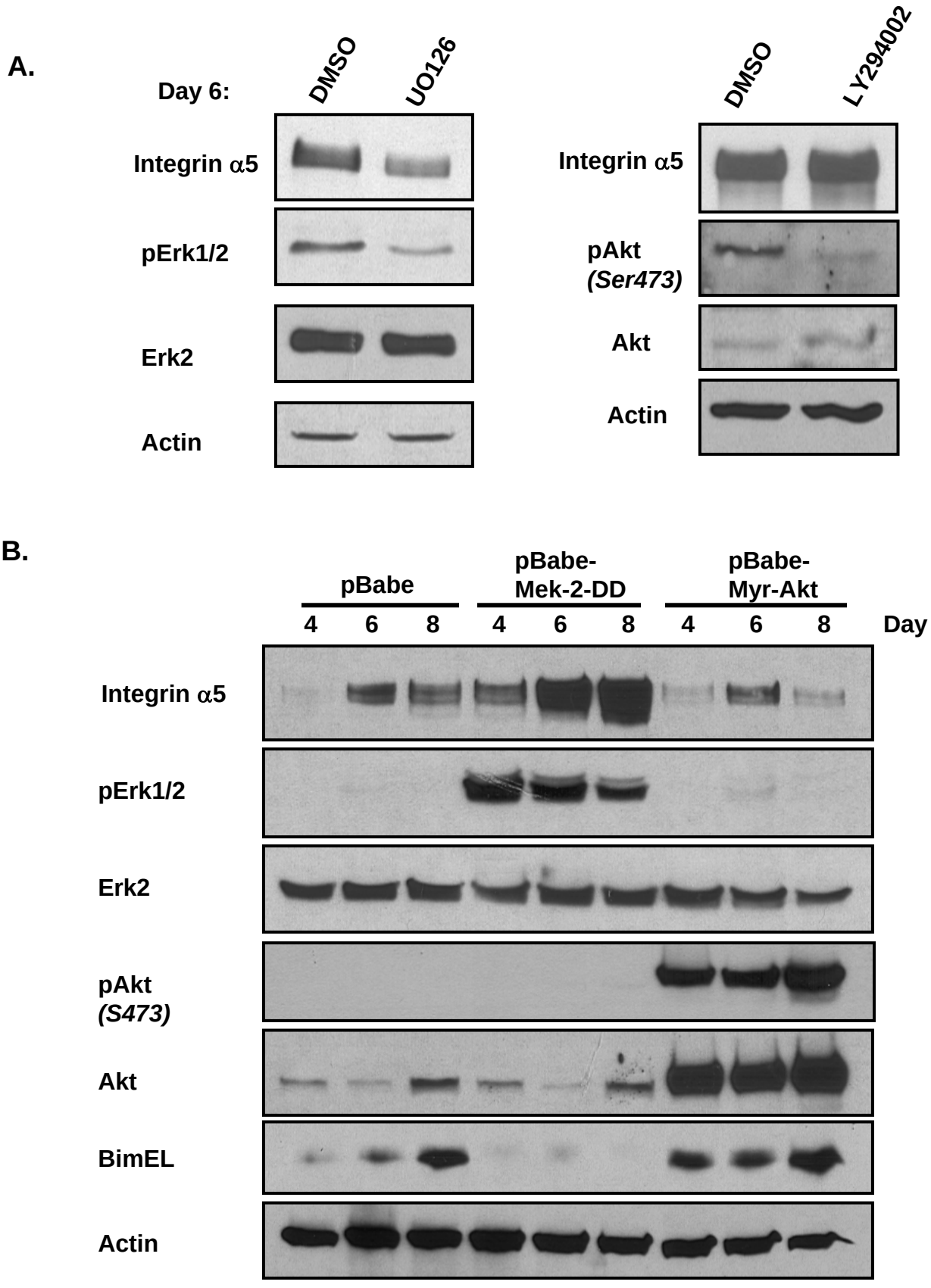
A.

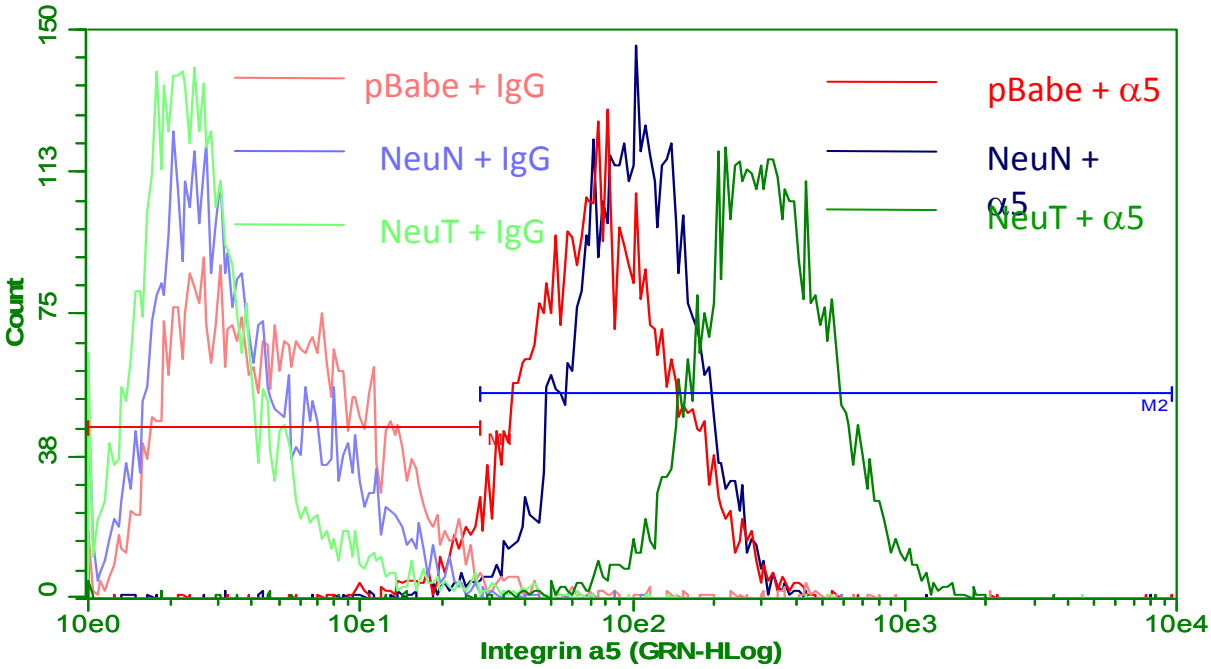

siRNA:
Cont
ITGα5 #1
ITGα5 #2
0
48
0
48
0
48
hrs susp
pMek
Mek1/2
pErk
Erk2
pAkt (S473)
Akt
pSrc (Y416)
Src
Actin

B.


Attached
Suspended 48hrs
siRNA:
Cont
ITGα5#1
ITGα5#2
Cont
ITGα5#1
ITGα5#2
pErbB2 (total pY)
pErbB2 (Y877)
pErbB2 (Y1248)
ErbB2
Actin


A.
DMSO-Day 12
PP2-Day 12
Act-Caspase-3
DAPI
Act-Caspase-3
DAPI
B.
DMSO
PP2
Percent Caspase-3 Positive (2 or more)
**
***
Day 10
Day 12
C.
DMSO
PP2
Days in 3D:
12
12
pSrc (Y416)
c-Src
pErbB2 (Y877)
ErbB2
BimEL
Actin
D.
MCF10A-ErbB2: 48hrs suspension
shRNA:
Cont
c-Src
pSrc (Y416)
c-Src
pErbB2 (Y877)
ErbB2
BimEL
Caspase 3
Actin
E.
% Annexin/PI Labelled Cells
**
Cont shRNA
c-Src shRNA


A.
Day 6:
DMSO
UO126
Integrin α5
pErk1/2
Erk2
Actin
DMSO
LY294002
Integrin α5
pAkt
(Ser473)
Akt
Actin
B.
pBabe
pBabe-
Mek-2-DD
pBabe-
Myr-Akt
4 6 8 4 6 8 4 6 8
Day
Integrin α5
pErk1/2
Erk2
pAkt
(S473)
Akt
BimEL
Actin



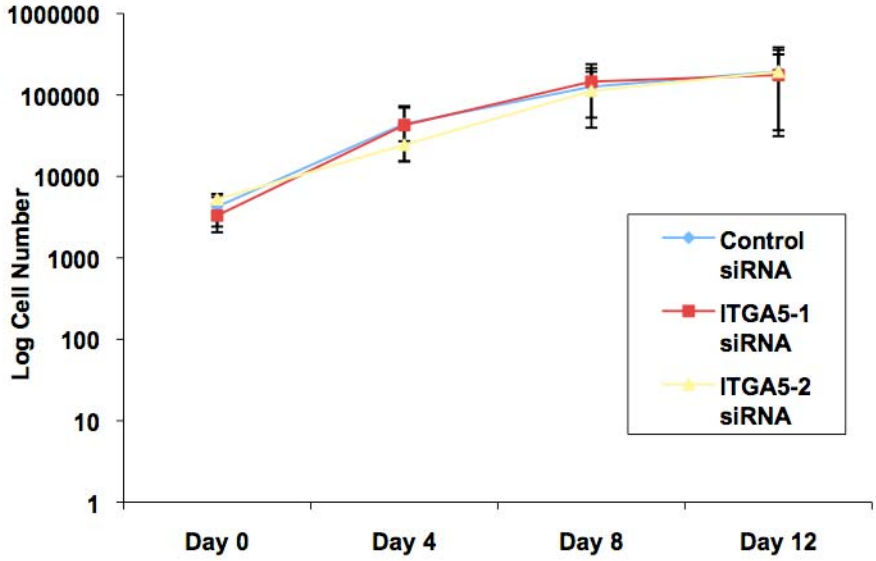
Supplementary Fig. S1. ErbB2 overexpression in MCF-10A cells induces increased expression of integrin α5. MCF-10A cells infected with retrovirus of empty vector (pBabe), wild-type ErbB2 (NeuN), or constitutively active ErbB2 (NeuT) were detached from plates using 5 mM EDTA, pH 7.4, and counted. Two million cells were incubated with N-terminus targeting anti-integrin α5 antibody (CD49e, BD Pharmingen); or isotype control Mouse IgG, (BD Pharmingen) at a 1:100 dilution, washed, and incubated with secondary antibody (AlexaFluor mouse 488, Invitrogen) at a 1:200 dilution. Cells were read on a Guava Technologies EasyCyte Plus, and data analyzed with Guava Cytosoft 5.3 software.

Table I. Information on HER2/ErbB2 and integrin α5 status.

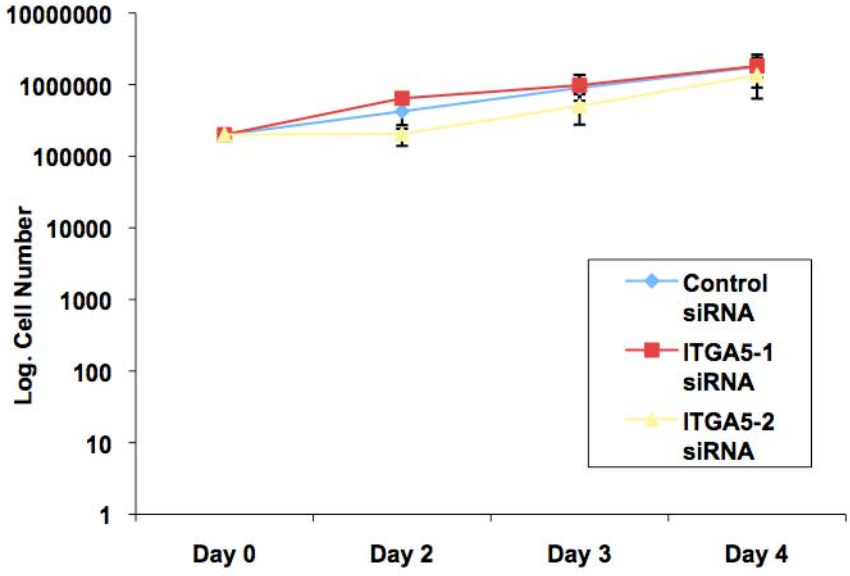
	Integrin α5 Negative		Integrin α5 Positive	
	n	%	n	%
Her2/ErbB2 status				
Negative	6	6.38	18	19.15
Positive	6	6.38	64	68.09

Table 1: Information on integrin α5 staining versus ErbB2/Her2 status obtained from tissue microarrays

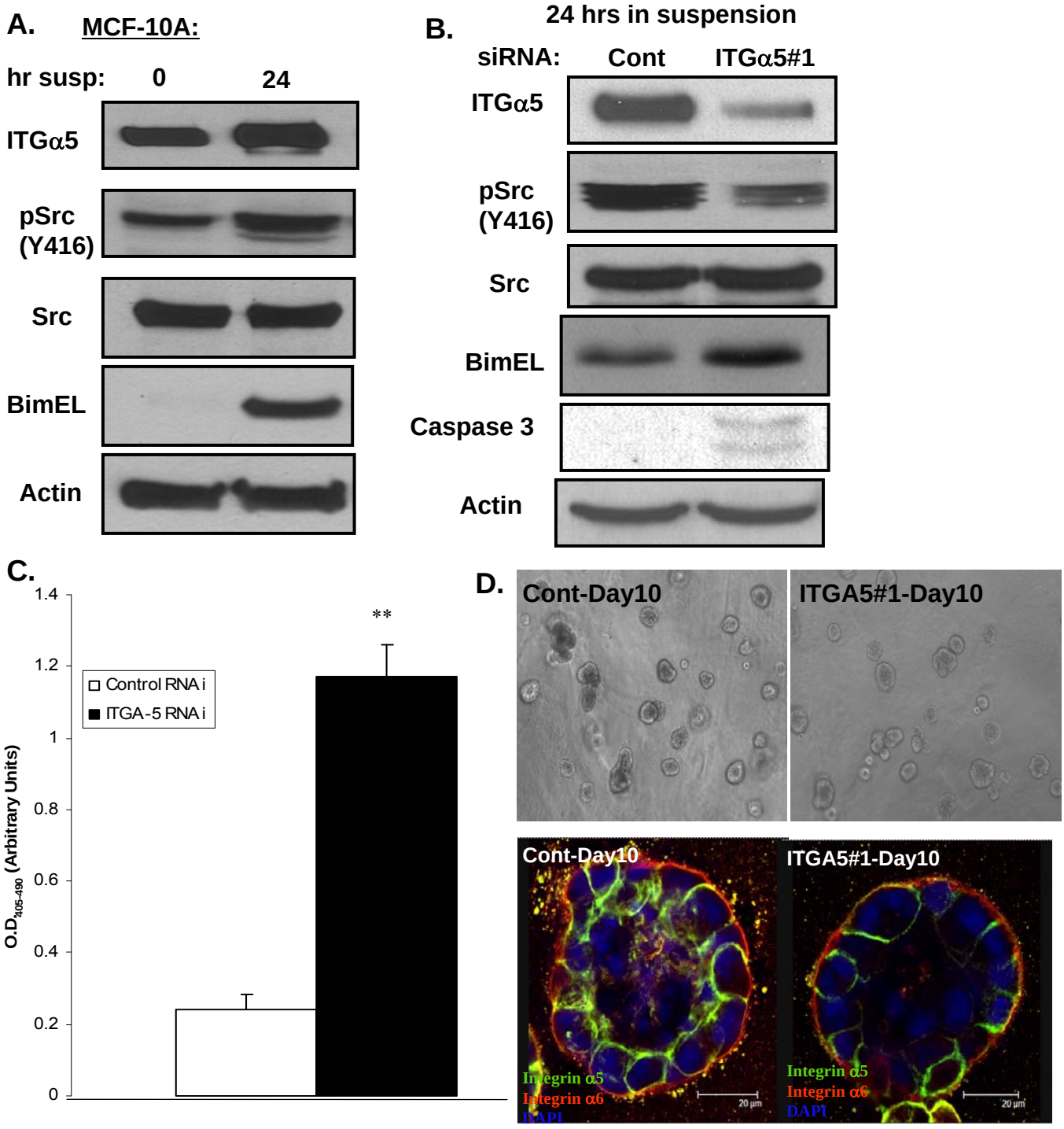
A. MCF-10A: NeuN: Growth in 3D



B. MCF-10A: NeuN: Growth in 2D

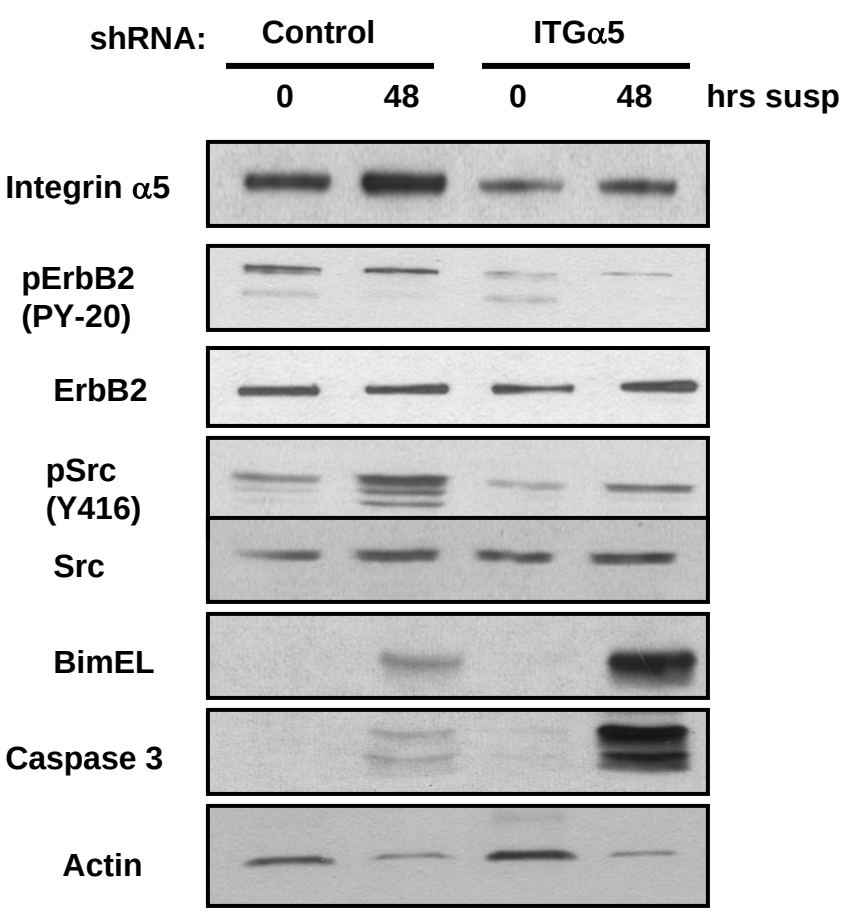


Supplementary Fig. S2. Targeting integrin α5 with siRNA does affect growth in 3D or in 2D (A) At 48 hours after initial transfection with siRNA oligonucleotides against integrin α5 or luciferase control, cells stably overexpressing NeuN were placed in 3D morphogenesis assay at 5000 cells/well. The cell number/well was then quantitated at day 0 (4 hours after seeding cells onto matrigel), day 4, day 8 and day 12. The graph shows an increase in cell number on a logarithmic scale plotted against days in 3D culture (n = 4, mean ± SD). Differences were not found to be statistically significant by Students *t* test. (B) Cells stably overexpressing NeuN were seeded at 200,000 cells/well onto 35mm tissue culture plates and monitored for growth during transfections with siRNA oligonucleotides against integrin α5 or luciferase control. The cell number/plate was then quantitated at day 0 (at time of seeding), day 2 (24hr after initial transfection), day 3 (48hr after initial transfection), day 4 (72hr after initial transfection). The graph shows an increase in cell number on a logarithmic scale plotted against time in days (n = 6, mean ± SD). Differences were not found to be statistically significant by Students *t* test.

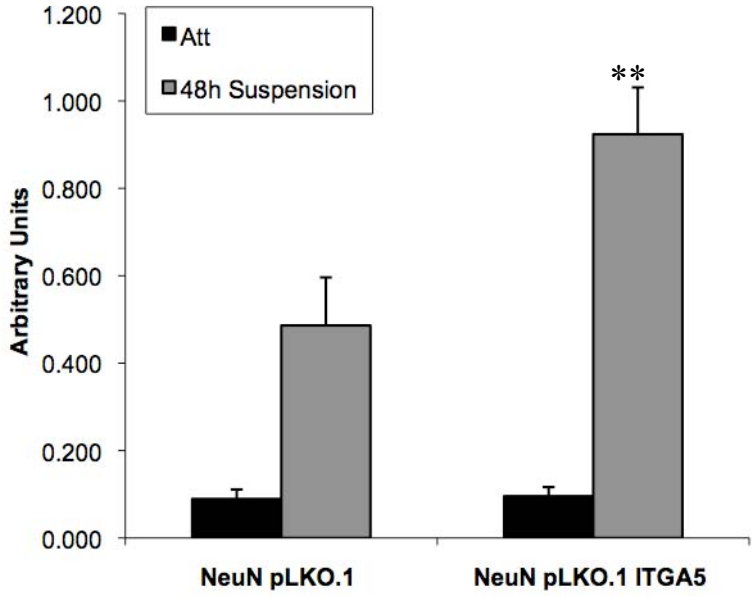


Supplementary Fig. S3. Targeting integrin α5 with siRNA sensitizes MCF-10A cells to anoikis but does alter acini formation in 3D. (A) Whole cell lysates prepared from MCF-10A cells that were either attached or cultured in suspension for 24 hours were analyzed by Western blot using antibodies against integrin α5, phospho-Src, Src, Bim and actin. (B-C) At 48 hours after initial transfection with siRNA oligonucleotides against integrin α5 (ITGA5 siRNA#1) or luciferase control, (B) MCF-10A cells were cultured in suspension for 24 hours followed by cell lysis and analysis by Western blot using the indicated antibodies or (C) placed in suspension in methylcellulose/media on poly-HEMA-coated plates for 24hr and then analyzed for apoptosis using colorimetric DNA Fragmentation ELISA (n=3, mean ± SD, **p<0.05 by Students *t* test). (D) siRNA transfected MCF-10A cells were also placed in 3D morphogenesis assays and were fixed and immunostained at day 10 with antibodies for anti-integrin α5 (green) and integrin α6 (red), and nuclei were counterstained with DAPI (blue). Images were captured using light microscope (top panel) and confocal microscopy (bottom panel).

A. MCF-10A: ErbB2 (NeuN)



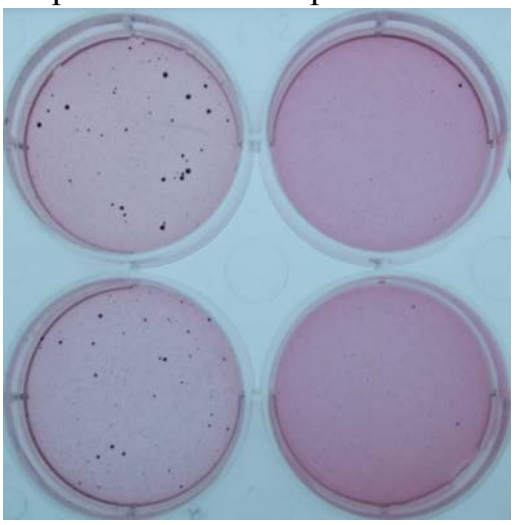
B. MCF-10A: ErbB2 (NeuN)



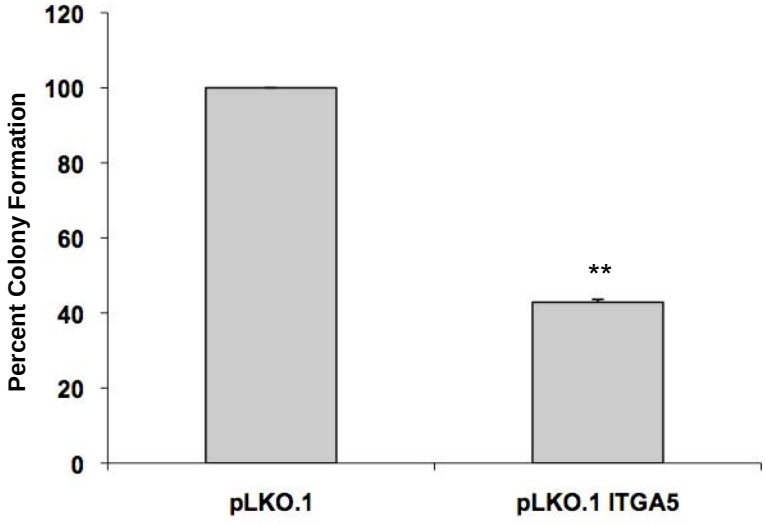
C.

MCF-10A: ErbB2 (NeuT)

pLKO.1 pLKO.1 ITGA5

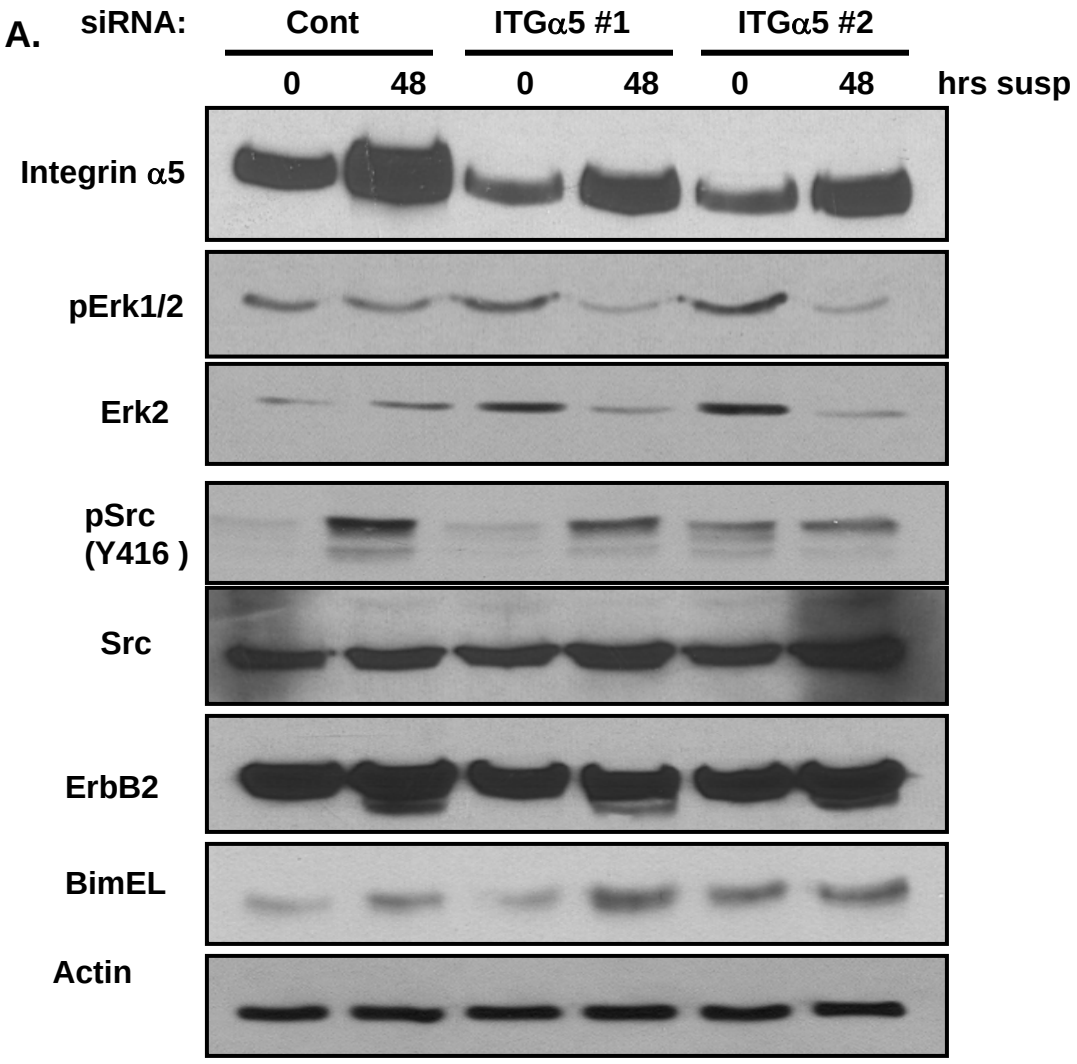


D. MCF-10A: ErbB2 (NeuT)

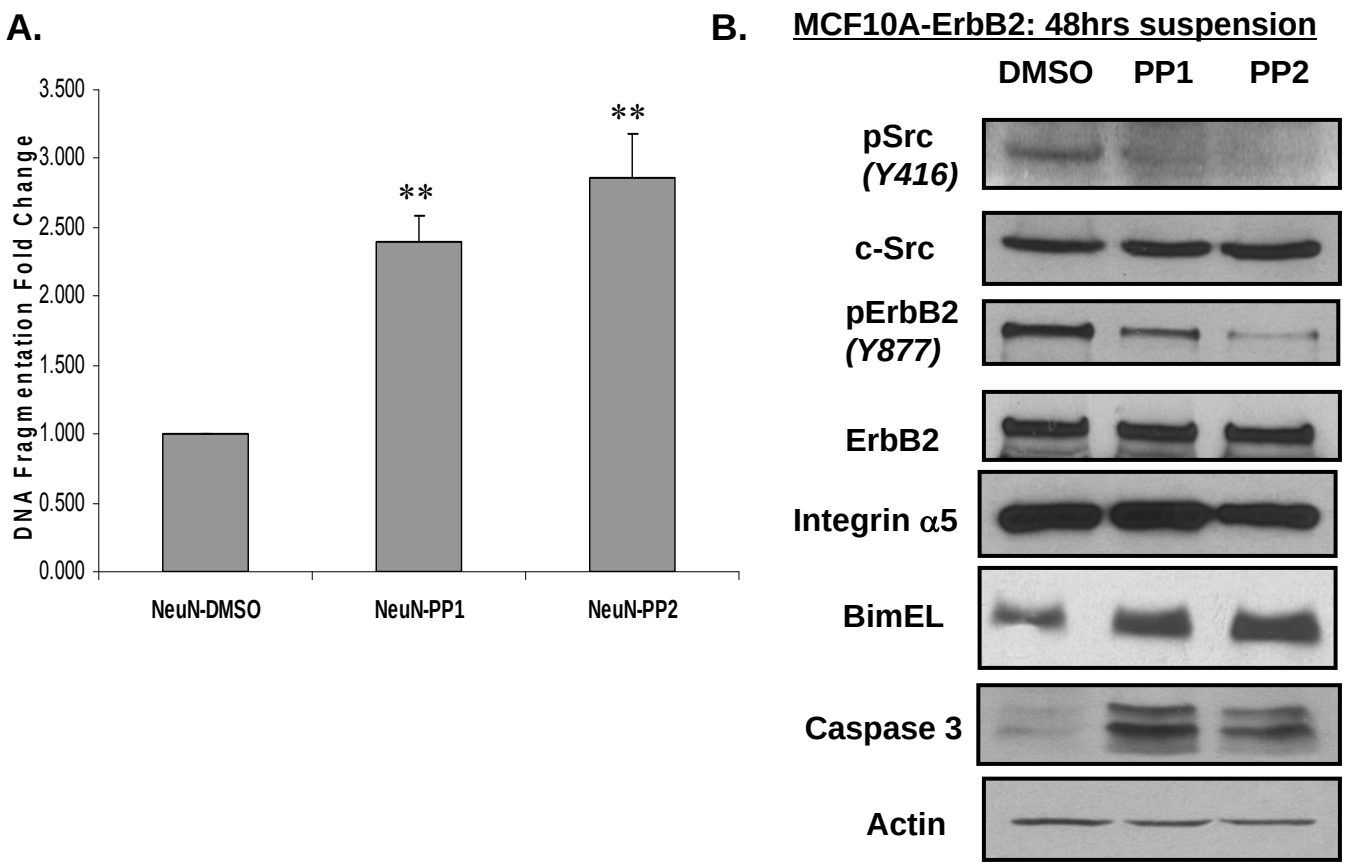


Supplementary Fig. S4. Targeting integrin α5 with shRNA reverses ErbB2-mediated anoikis resistance, Bim inhibition, and anchorage independence. (A-B) MCF-10A cells stably overexpressing wild-type ErbB2 (NeuN) were infected with lentivirus carrying shRNA constructs against integrin α5 or scrambled sequence. (A) Cells were lysed from monolayer culture or after 48hr suspension culture on poly-HEMA-coated plates, and proteins analyzed by immunoblotting with antibodies to integrin α5, phospho-ErbB2 (PY-20), ErbB2, phospho-Src, Src, Bim, activated caspase-3 and actin. (B) Cells from above were placed in suspension in methylcellulose/media on poly-HEMA-coated plates for the indicated times and then used to perform a DNA Fragmentation ELISA. Values represent the mean ± SD of A405 for at least three independent experiments (**$p<0.05$ by Students *t* test for comparing 48h suspension samples). (C-D) MCF-10A cells stably overexpressing activated ErbB2 (NeuT) were infected with lentivirus carrying shRNA constructs against integrin α5 or control (scrambled) sequence, and then placed in soft agar plates for 21 days followed by staining with INT violet. Representative plates were imaged(C), and the number of soft agar colonies were counted and normalized and compared to that of control shRNA (D). The values shown represent averages of normalized values (n = 3, mean ± SE (**$p<0.0001$ by Students *t* test)).

MCF-10A: ErbB2 (NeuT)



Supplementary Fig. S5. Integrin α5 is required for signaling from oncogenic ErbB2. MCF-10A cells stably overexpressing activatedErbB2 (NeuT) were transfected with luciferase control or integrin α5 siRNA oligos. At 48 hours after initial transfection cells were placed in suspension culture on poly-HEMA-coated plates for indicated times before lysis. Proteins were analyzed by immunoblotting with the indicated antibodies.



Supplementary Fig. S6. Src Regulation of ErbB2-mediated Anoikis Resistance. (A) MCF-10A cells stably overexpressing NeuN were cultured in suspension in methylcellulose/media on poly-HEMA-coated plates for 48hr in the presence of DMSO vehicle control, PP1 (10μM) or PP2 (10μM). Cells were then used to perform a DNA Fragmentation ELISA. Apoptosis of each sample was normalized and compared to control (DMSO) and represented as fold increase in DNA fragmentation (A_{405}). The values shown represent averages of normalized values (n = 3, mean ± SE (**p<0.05 by Students *t* test)). (B) NeuN cells were cultured in suspension for 48hr on poly-HEMA-coated plates in the presence of DMSO vehicle control, PP1 (10μM) or PP2 (10μM) followed by cell lysis. Proteins were analyzed by immunoblotting with antibodies to phospho-Src, c-Src, phospho-ErbB2 (Y877), ErbB2, integrin α5, bim, activated (cleaved) caspase-3, and actin.

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Publications

Keneshia K. Haenssen, Sarah A. Caldwell, Kristina S. Shahriari, S. RaElle Jackson, Kelly A. Whelan, Andres J. Klein-Szanto and Mauricio J. Reginato. ErbB2 requires integrin α5 for anoikis resistance via c-Src regulation of receptor activity in human mammary epithelial cells *(Journal of Cell Science*, under review*)*

Temitope R. Sodunke, **Keneshia K. Turner**, Sarah A. Caldwell, Kevin W. McBride, Mauricio J. Reginato and Hongseok "Moses" Noh. Micropatterns of Matrigel for three-dimensional epithelial cultures. ***Biomaterials*** 2007.Vol 28 (27) 4006-4016

Abstracts and Presentations

Haenssen, K. K., Caldwell, S. A., Shahriari, K., and Reginato, M. J. Requirement of Integrin α5 for Survival of ErbB2/Neu Expressing Mammary Epithelial Cells. CSHL: Mechanisms and Models of Cancer Meeting. August 2008.

Haenssen, K. K., Caldwell, S. A., Shahriari, K., Jagtap, S., and Reginato, M. J. Requirement of Integrin α5 for Survival of Normal and ErbB2/Neu Expressing Mammary Epithelial Cells. AACR: Frontiers in Cancer Prevention Meeting. December 2007.

Turner, K. K., Caldwell, S. A., and Reginato, M. J. Requirement of Integrin α5 for Survival of Normal and ErbB2/Neu Expressing Mammary Epithelial Cells. Gordon Research Conference: Signaling by Adhesion Receptors. June 2006.

Turner, K. K., Caldwell, S. A., and Reginato, M. J. ErbB2/Neu Upregulates and Requires Integrin α5 for Anchorage-independence of Mammary Epithelial Cells. AACR: Molecular Targets and Cancer Therapeutics Meeting. November 2005.

Turner, K. K., Caldwell, S. A., and Reginato, M. J. Role of Integrin α5 in Mammary Morphogenesis, Survival, and Oncogenesis. Discovery Day, Drexel University College of Medicine, October 2005 (oral presentation).

Grants and Awards

Department of Defense Breast Cancer Predoctoral Fellowship, (2008-2010)-Funds to cover stipend for two years of breast cancer research under Mentor Mauricio J.Reginato, Ph.D.

International Grant, Randolph-Macon Woman's College (1999-2003)-Award covering 63% of tuition and living expenses for four years.

Founder's Fellowship, Randolph-Macon Woman's College (1999-2003)-Award covering 37% of tuition and living expenses for four years.

Honors

Honorable Mention for Oral Presentation, DUCOM Discovery Day (2005): Title: "Role of Integrin α5 in Mammary Morphogenesis, Survival, and Oncogenesis." under Mentor Mauricio J.Reginato, Ph.D.

Professional Service

Molecular Cell Biology and Genetics Program Seminar Planning Committee, DUCOM (Jan 2007-Dec 2008)